

GLUTAMINE METABOLISM IN  
NORMAL AND ACIDOTIC RATS  
IN VIVO

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GLUTAMINE METABOLISM IN NORMAL AND ACIDOTIC RATS IN VIVO

By

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A Thesis submitted in partial fulfillment of the  
requirements for the degree of Master of Science

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## ABSTRACT

The kidney responds to metabolic acidosis by the increased excretion of the ammonium salts of nonvolatile acids in the urine. It is therefore important to maintain an adequate supply of precursors of urinary ammonia in order to maintain acid-base balance. In this work, arterio-venous differences for all amino acids were measured across the kidneys of normal and acidotic rats to determine which amino acids were important in urinary ammonia production. Glutamine was the only amino acid taken up by the acidotic kidney; no amino acids were removed by the normal kidney.

These measurements were made using whole blood since amino acid levels in whole blood and plasma were found to differ. Glycine, glutamate, lysine, and arginine were concentrated within the blood cells. In addition, levels of serine, threonine and lysine rose and levels of glutamine fell in acidosis in both whole blood and plasma. Whole blood and plasma arterio-venous differences were measured for glutamine across the acidotic rat kidney. Results showed that glutamine is removed exclusively from the plasma.

In a further study on kidney metabolism, arterio-renal venous differences for lactate were measured. Since both normal and acidotic kidney removed equivalent amounts of lactate, it was concluded that the oxidation of lactate carbon was not replaced by the oxidation of glutamine carbon in acidosis.

The site of production of glutamine and the changes which occur in glutamine metabolism in response to an increased acid load were then in-



vestigated. To this end, measurements of the turnover rate of free glutamine in the body were made using both the single injection and constant infusion methods. However, in order to obtain accurate estimates of the turnover rate, a technique for the measurement of the specific activity of 1-<sup>14</sup>C-glutamine first had to be devised. Results obtained by both methods showed that the turnover rate of glutamine did not change in acidosis. This was taken to indicate that the extra glutamine required by the kidney in acidosis was supplied by a lessening of glutamine utilization by other tissues. Turnover values obtained by the single injection method were considerably higher than those obtained by constant infusion. The pool size of glutamine calculated from the single injection data was also considerably smaller than the total body glutamine pool obtained by analysis of whole body extracts and by a summation of data from other investigators. These differences were thought to be due to the methods used in the analysis of the single injection data.

Arterio-venous differences for glutamine were measured across the major tissues of the body in order to determine the site of glutamine production. Although glutamine was produced by the muscle of the normal rat, no significant output of glutamine by any tissue was found in acidosis. The kidneys and gastrointestinal tract removed glutamine during acidosis. Arterio-venous differences were also measured for glutamate to find if glutamate uptake paralleled glutamine output. However, glutamate was released by the muscle and brain of the normal rat and by the gastrointestinal tract of the acidotic rat; no uptake of glutamate was found. It thus appears that the glutamate required for glutamine synthesis does not come directly from the blood.

## ACKNOWLEDGEMENTS

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## LIST OF ABBREVIATIONS

ALA	alanine
ANSER	anserine
ARG	arginine
ASN	asparagine
ATP	adenosine triphosphate
A-FV	arterio-femoral vein difference
A-HV	arterio-hepatic vein difference
A-JV	arterio-jugular vein difference
A-PV	arterio-portal vein difference
A-RV	arterio-renal vein difference
CARN	carnosine
CIT	citrulline
GDC	glutamate decarboxylase
G.I.T.	gastrointestinal tract
GLN	glutamine
GLU	glutamate
GLY	glycine
HIS	histidine
ILE	isoleucine
LEU	leucine
LYS	lysine
MET	methionine
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)

OHPRO	hydroxyproline
ORN	ornithine
PHE	phenylalanine
PRO	proline
SER	serine
TAU	taurine
THR	threonine
TYR	tyrosine
VAL	valine

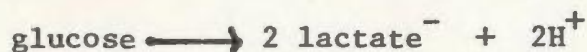


## INTRODUCTION

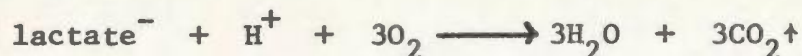
### I. GENERAL

#### A. Production of Metabolic Acid

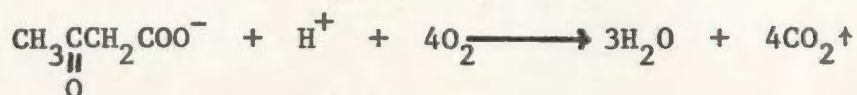
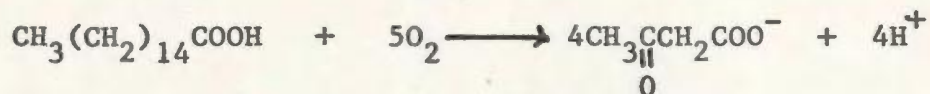
One of the most important physiological problems faced by animals is the maintenance of acid-base homeostasis. To achieve this end, animals must ultimately match their metabolic processes so that the overall result is neither the production nor the uptake of excess acid. Thus, the oxidation of glucose to lactate:



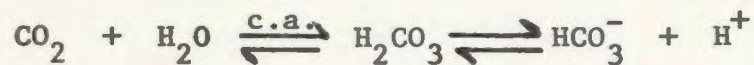
may be coupled to the oxidation of lactate to  $\text{CO}_2$  and water:



Similarly, the oxidation of fat to acetoacetate is normally coupled to the oxidation of acetoacetate to  $\text{CO}_2$  and water:



However, the matching of metabolic processes to maintain neutrality is not always possible. For example, the production of  $\text{CO}_2$  by the tissues actually results in the production of acid as long as the  $\text{CO}_2$  produced remains in an aqueous environment:



The formation of carbonic acid from  $\text{CO}_2$  and  $\text{H}_2\text{O}$  is catalyzed by the

enzyme carbonic anhydrase (c.a.).  $\text{CO}_2$  also reacts with undissociated aliphatic amino groups to form carbamino compounds with a concomitant production of acid:



Thus, a small amount of  $\text{CO}_2$  is bound to plasma proteins, this amount being of little consequence in the transport of  $\text{CO}_2$  from the tissues. However, the increased  $\text{pCO}_2$  of venous blood results in a three-fold increase in the amount of carbamino hemoglobin in venous blood which would significantly increase the acidity of the blood were it not for other considerations.

Other mechanisms exist which produce excess acid in the body. The catabolism of sulfur-containing amino acids, including methionine and cystine, results in the production of sulfuric acid. Breakdown of organically bound phosphate (e.g., phospholipid) causes the release of  $\text{H}_2\text{PO}_4^-$  and a proton.

#### B. Buffers of the Body

Excess acid produced is buffered in the blood mainly by hemoglobin, the imidazole group of histidine residues being chiefly responsible for the buffering action. However, oxygenated hemoglobin ( $\text{HbO}_2$ ) is a stronger acid than deoxygenated hemoglobin (Hb). Thus, not only is hemoglobin a good buffer when oxygenated, but when it loses its oxygen and picks up  $\text{CO}_2$ , its buffering action is increased. In this way, the pH values of arterial and venous bloods usually differ by no more than 0.03 pH units.

The major buffer of the extracellular fluid is the  $\text{HCO}_3^-$ - $\text{H}_2\text{CO}_3$  system which works mainly in conjunction with hemoglobin to maintain a constant body pH. Although the  $\text{HCO}_3^-$ - $\text{H}_2\text{CO}_3$  buffer system has a pKa of only 6.1, it is an effective buffer at physiological pH values, due to the constant  $\text{pCO}_2$  (about 40 mm Hg) in the alveolar air. Thus, the  $[\text{H}_2\text{CO}_3]$  is fixed by the constant  $\text{pCO}_2$  and only the  $[\text{HCO}_3^-]$  varies with any added acid or alkali. Thus, the  $\log \frac{[\text{HCO}_3^-]}{\text{H}_2\text{CO}_3}$  term of the Henderson-Hasselbalch

equation (and consequently the pH) will not change as readily as it would for a buffer with no fixed components. The buffering efficiency of the  $\text{HCO}_3^-$ - $\text{H}_2\text{CO}_3$  system is further complemented by the presence of hemoglobin in erythrocytes. Since some acid is buffered by hemoglobin, more  $\text{HCO}_3^-$  can remain in plasma and be available to buffer strong acids.

### C. Regulation of $\text{HCO}_3^-$ - $\text{H}_2\text{CO}_3$ System

The  $\text{pCO}_2$  of the air in the lungs is dependent on the rate and depth of respiration, which is controlled by a respiratory centre in the nervous system. This area has sensors which are sensitive to changes in the pH and  $\text{pCO}_2$  of the extracellular fluid. Thus, a fall in pH of the extracellular fluid due to diminished  $[\text{HCO}_3^-]$  causes a stimulation of respiration and consequently a lowering of aveolar  $\text{pCO}_2$ . Hence, extracellular  $[\text{H}_2\text{CO}_3]$  is also lowered, and a rise in pH back to the normal of 7.4 results. A rise in plasma pH or plasma  $[\text{HCO}_3^-]$  causes the opposite effects. Pulmonary regulation of  $[\text{H}_2\text{CO}_3]$  is extremely rapid although compensation is never complete, since a return of  $[\text{H}_2\text{CO}_3]$  levels to normal



reverses the effects on the respiratory centre.

The kidney regulates the pH of body fluids by controlling the  $[\text{HCO}_3^-]$  of the blood. Renal compensation requires longer periods of time than pulmonary compensation to be effective but may result in complete restoration of normal pH and bicarbonate levels. A fall in pH due to increased aveolar  $\text{CO}_2$  or decreased  $[\text{HCO}_3^-]$  is counteracted by the excretion of acidic urine which is buffered by ammonia. An increase in pH of the extracellular fluid results in the excretion of  $\text{Na}^+$ ,  $\text{HCO}_3^-$  and the dissociated forms of other weak acids. Normally however the kidney reabsorbs 99.9% (some 5,100 meq/day in man) of the bicarbonate in the glomerular filtrate. Bicarbonate that was used to neutralize excess acid in the blood is regenerated in the kidney tubular cells.

The mechanism of reabsorption of bicarbonate, excretion of acid urine and excretion of ammonia is outlined in Fig. 1 and Fig. 2. According to this mechanism, carbonic acid generated within the cell by the action of carbonic anhydrase (c.a.) dissociates to give bicarbonate ion and a proton. This proton is actively exchanged for a sodium ion in the tubular urine. Sodium bicarbonate is then removed from the cell into the blood. Acid is then either excreted directly as phosphate salts (mainly  $\text{NaH}_2\text{PO}_4$ ) (Fig. 2) called "titratable acidity" or is excreted in combination with ammonia, produced within the cell from amino acid precursors, designated in the figure, "X" (Fig. 1). Only a limited amount of titratable acid can be excreted, as the maximum  $\text{H}^+$  gradient attainable across the tubular cell membrane limits the pH of the urine to around 4.4. Also, the excretion of titratable acidity in large amounts would

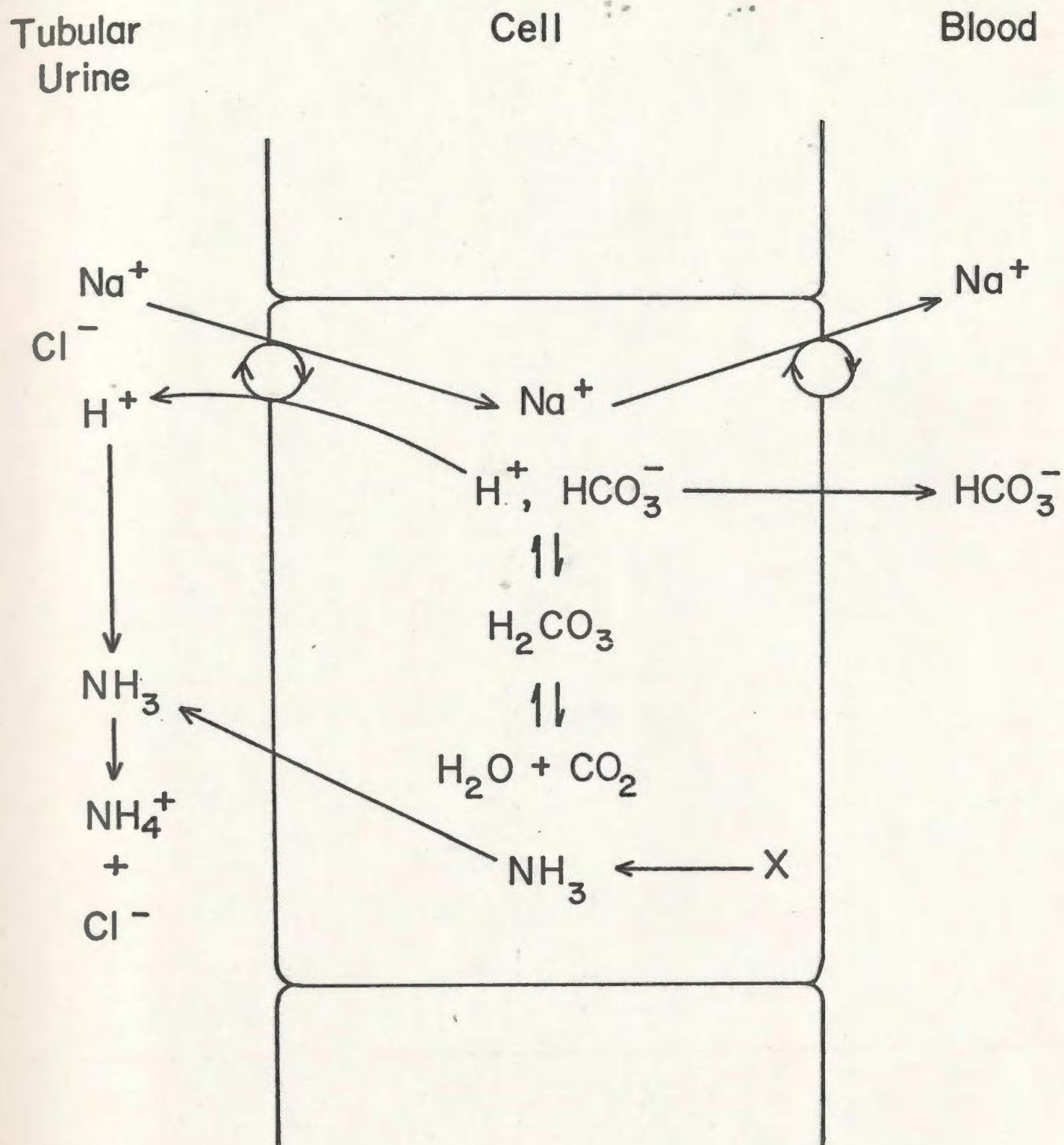


FIG. 1. Mechanism of the Excretion of Ammonia.

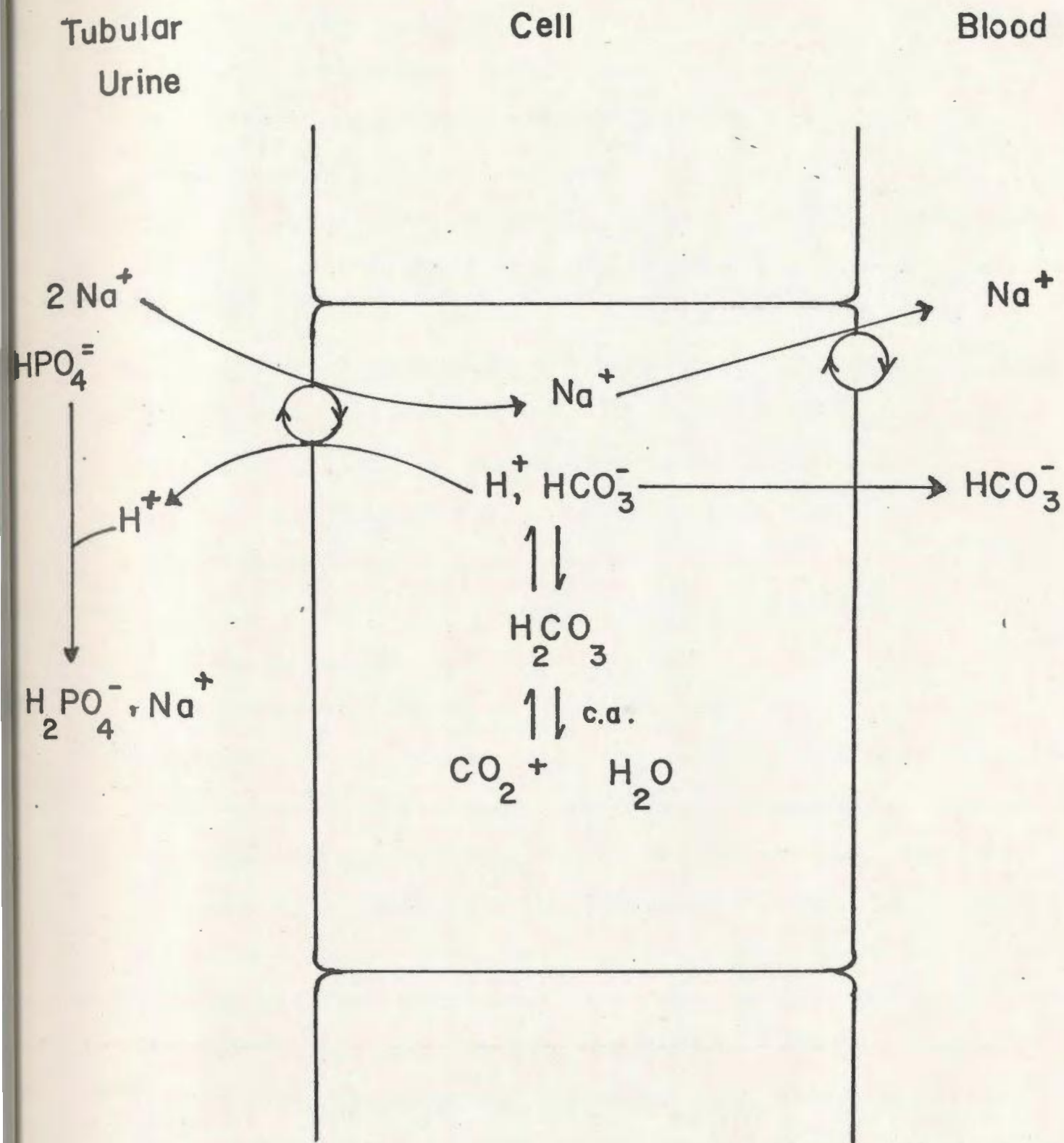


FIG. 2. Mechanism of the Excretion of Titratable Acidity.



seriously deplete extracellular sodium supplies. Ammonia excreted in the urine thus salvages vital cations as well as neutralizes excess acid.

Ammonia is thus an extremely important buffer of urine acid but it is not a mechanism for the elimination of waste nitrogen in higher animals. It has several advantages that make it particularly suitable as a buffer in urine: (1) it is readily available through deamination and deamidation of amino acids and its formation does not directly utilize ATP, (2) in its unprotonated form ( $\text{NH}_3$ ), it is lipid soluble and readily diffuses across tubular cell membranes. In the acidic urine it becomes protonated ( $\text{NH}_4^+$ ) and is then lipid insoluble. This process whereby ammonia moves into the urine and is then trapped there is referred to as "diffusion trapping".

#### D. Acidosis and Alkalosis

Although normally the mechanisms that produce acid closely match those mechanisms that consume acid, the production of acid may be too great in certain circumstances. For example, in diabetes mellitus the breakdown of fats occurs very rapidly and leads to hepatic conversion of acetyl CoA to acetoacetic and  $\beta$ -hydroxybutyric acids (ketone bodies). A buildup of lactic acid in muscle may occur during severe exercise due to temporary oxygen deficiency. This buildup of nonvolatile strong acids in the blood may cause a lowering of blood pH and  $[\text{HCO}_3^-]$  levels, a condition referred to as metabolic acidosis. Metabolic acidosis may also be caused by a loss of bicarbonate from the intestine or via the kidneys during renal failure. Experimental acidosis is often attained by the ad-

ministration of acid by stomach tube, or more commonly, by the ingestion of  $\text{NH}_4\text{Cl}$ .  $\text{NH}_4\text{Cl}$  dissociates to form  $\text{NH}_3$  and  $\text{HCl}$ , the ammonia being removed by the liver to form urea. Metabolic alkalosis is characterized by a rise in pH due to buildup of alkali in the blood as may be caused by the metabolism of the anions of weak acids as found in fruits and vegetables, or by the ingestion of excess  $\text{NaHCO}_3$ .

Due to respiratory problems, alterations in  $[\text{H}_2\text{CO}_3]$  may also occur which affect acid-base status. Pneumonia, pulmonary edema and other causes of respiratory difficulty may result in increased  $\text{pCO}_2$  of alveolar air and a concomitant rise in  $[\text{H}_2\text{CO}_3]$  and a lowering of plasma pH. Increased plasma  $[\text{HCO}_3^-]$  then results due to buffering of  $\text{H}^+$  by hemoglobin. Thus, a low plasma pH with both elevated  $[\text{H}_2\text{CO}_3]$  and elevated  $[\text{HCO}_3^-]$  is observed and this condition is referred to as respiratory acidosis. Conversely, respiratory alkalosis is a result of hyperventilation of the lungs, causing a lower  $\text{pCO}_2$  and  $[\text{H}_2\text{CO}_3]$  and thereby elevating pH. Since arterial  $\text{pCO}_2$  is low, buffering of the  $\text{H}^+$  by hemoglobin automatically decreases plasma  $[\text{HCO}_3^-]$  in an attempt at preventing the rise in plasma pH.

## II. SOURCES OF URINARY AMMONIA

### A. Important Amino Acid Precursors

Since ammonia is such an important buffer in urine, allowing the excretion of acid without the loss of vital cations, its precursors and the source of its precursors are of great interest. At first it was be-



lieved that ammonia removed by the kidney was preformed in blood. In 1921, Nash and Benedict (54) produced the first evidence that ammonia preformed in the blood was not the major source of urinary ammonia. They cited low arterial levels of free  $\text{NH}_3$ , which remained unchanged in acidosis, alkalosis, and after nephrectomy, as well as a renal venous  $\text{NH}_3$  level that was twice as high as the arterial level. They thus concluded that  $\text{NH}_3$  was formed within the kidney from nitrogenous precursors. Although they conceded that amino acids may be the precursors of urinary ammonia, they favored the idea that urea was the major precursor.

It was not until 1943 that Van Slyke et al. (77) reported the first evidence that glutamine was the major precursor of urinary ammonia. Using the dog, kidneys were exteriorized to allow removal of blood samples from the renal vein without anaesthesia. They found that all urea removed by the kidneys was excreted unchanged in the urine even during metabolic acidosis. Glutamine taken up did not appear in the urine. Glutamine was taken up in such amounts that the amide nitrogen alone could account for all of the ammonia put out in the renal vein as well as 60% of the ammonia in the urine. Glutamine extraction was decreased during infusion of  $\text{NaHCO}_3$ , while glutamine administered to an acidotic dog markedly increased ammonia excretion.

A different approach was used by Davies and Yudkin (22) to determine which amino acids were important in the production of urinary ammonia. Using rats made chronically acidotic (by feeding 0.05 M HCl) or alkalotic (by feeding 0.1 M  $\text{NaHCO}_3$ ) for 3 to 8 months, ammonia production by kidney slices was measured using L-glutamate, glycine, L-leucine, L-aspartic acid or L-alanine. From these measurements, the involvement of



glutaminase, glycine oxidase, L-amino acid oxidase and the transaminase enzyme systems was inferred. These authors found increased ammonia production during acidosis and decreased ammonia production during alkalosis from L-glutamine, glycine and L-leucine, and no change in ammonia production from L-aspartate and L-alanine. They thus suggest that urinary ammonia is produced by deamination of glutamine, deamination of glycine by glycine oxidase, and deamination of other monoamino monocarboxylic acids by L-amino acid oxidase.

Following the development of the automatic amino acid analyzer, Owen and Robinson (56) measured the extraction of amino acids by the human kidney. They found that glutamine was the major amino acid extracted by the normal kidney ( $47 \pm 14$   $\mu$ moles/min); the extraction of glutamine by the kidney was greatly enhanced ( $102 \pm 53$   $\mu$ moles/min) in acidosis. The rate of glutamine extraction may not, however, account for all the ammonia produced by the kidney. If only the amide nitrogen of glutamine was considered in ammonia production, only 63% of the total  $\text{NH}_3$  production of the normal kidney and 74% of the total  $\text{NH}_3$  produced by the acidotic kidney could be accounted for. Small uptakes of proline, glycine and possibly valine were thought to account for the balance of the  $\text{NH}_3$  produced by the normal kidney. These amino acids may also contribute to the ammonia production by the acidotic kidney. There was a substantial output of serine ( $30 \pm 27$   $\mu$ moles/min) as well as outputs of alanine, cysteine and arginine by both normal and acidotic kidneys. However, while glutamate was released by the normal kidney, it was taken up by the acidotic kidney. It thus seems probable

that the  $\alpha$ -amino nitrogen of glutamine is used in the production of substantial amounts of ammonia by the acidotic human kidney while it may not contribute to ammonia production by the normal kidney.

In the same year, Shalhoub et al. (71) measured arterio-venous differences for some 23 amino acids in plasma across the kidneys of dogs. Acute metabolic acidosis was first induced in these animals by the administration of 10 g  $\text{NH}_4\text{Cl}$  orally for 3 days and then acute alkalosis was induced by intravenous infusion of 1%  $\text{NaHCO}_3$ . Results were similar to those already described in man (56). In acidosis, glutamine plus asparagine (glutamine was not separated from asparagine by these authors), glycine, citrulline, tryptophan and proline are extracted by the kidney while alanine, serine, glutamate, cystine and ornithine are added to the renal venous plasma. The very low output of glutamate by the acidotic kidney suggest again that both the amide and  $\alpha$ -amino nitrogens of glutamine are used in ammonia production during acidosis. When acute metabolic alkalosis was induced, the extraction of glutamine plus asparagine was halved, while the extraction of glycine and addition of serine and alanine remained unchanged. Thus, with the induction of acidosis in the dog as well as the human, increased extraction and breakdown only of glutamine results in the greatly increased rate of ammonia excretion.

Further experiments from the same laboratory (19) tested the effects of infusion of ammonia, amides and amino acids into one renal artery of the dog on the ammonia excretion of both kidneys. Infusion of ammonium lactate into one renal artery resulted in increased ammonia output by that



kidney. Infusion of glutamine, asparagine, alanine, leucine or glycine, however, resulted in increased ammonia excretion by both kidneys due to a significant buildup of the infused amino acid in the general circulation. Previous experiments of this type have been summarized (10) and amino acids have been classified according to their potency for increasing urinary ammonia output. The physiological significance of these types of loading experiments is questionable due to the high concentrations of amino acids used. These high levels may cause amino acids to be utilized that would not normally be involved in ammonia production. This is borne out by experiments where infusion of D-amino acids resulted in increased ammonia production, while D-amino acids do not occur in significant amounts in the blood of animals. In addition, while alanine was found to contribute to urinary ammonia when administered in large amounts (19), in the intact animal (71) alanine is generally released by the kidney and thus cannot be an important precursor of urinary ammonia.

Two years later, Pitts et al. (62) used a new technique to determine exactly which amino acids contribute to the production of ammonia in the acutely acidotic dog. They infused  $N^{15}$ -labelled amino acids into the left renal artery of the dog and measured the production of  $N^{15}$  ammonia by the left kidney.  $N^{15}$  ammonia produced by the right kidney was due to recirculated  $N^{15}$ -label and was therefore subtracted from the  $N^{15}$  ammonia produced by the left kidney. From these studies, it was determined that the amide of glutamine contributes 33 to 50% of urinary ammonia production, the  $\alpha$ -amino nitrogen of glutamine contributes 16 to 25%, the amino of glycine contributes 3.1 to 4.3% and the amino nitrogen



of glutamate contributes 1.4 to 2.4%. The finding that the amino nitrogen of glutamate is not a good precursor of urinary ammonia although the amino nitrogen of glutamine is an important precursor is significant and consistent with previous in vivo studies. This may be due to either (1) a slow transport of glutamate into the mitochondria for deamination or (2) the possibility that glutamate is not produced in a free state following deamidation of glutamine, (3) the low physiological concentration of glutamate, although loading experiments have also shown glutamate to be ineffective in increasing urinary ammonia production.

A major problem with the  $N^{15}$  method is due to the necessity for about 50  $\mu$ moles of ammonia for the satisfactory determination of  $N^{15}$  by mass spectrometry. Thus, elevated levels of some amino acids are required to produce sufficient ammonia for  $N^{15}$  determination. Although infusion of  $N^{15}$  glutamine elevated arterial levels only 5-11%, the infusions of glycine and glutamate had to be at such a rate as to elevate arterial levels by 25% and 186-223% respectively. Utilization of amino acids at such elevated levels may have no physiological significance as discussed previously for the loading experiments. Care must be taken also when estimating the pathway of breakdown of the amino acids. It may not be possible to decide if deamination occurs directly by L-amino acid oxidase, as many transaminases exist within the cell which can transfer the amino group to a keto acid to form the corresponding amino acid which can then undergo deamination to release ammonia.

Work on the dog was continued by Addae and Lotspeich (2) who measured extrarenal glutamine arterio-venous differences. They found

that in acute sulfuric acid acidosis there is increased extraction of glutamine by parts of the intestinal tract as well as the brain. The liver, which normally puts out glutamine, makes more glutamine in the acutely acidotic dog to maintain a constant or slightly elevated arterial glutamine level. They found no significant arterio-venous difference for glutamine across the hindlimb in either the normal or acidotic dog. Measurements of glutamine content of various tissues in the rat showed that while glutamine utilizing tissues had lowered glutamine levels, the liver of the rat showed increased glutamine content after  $\text{NH}_4\text{Cl}$  administration. Also citing the work of Duda and Handler (25) who showed that  $\text{N}^{15}$ -label injected as ammonium lactate first appeared in the liver glutamine fraction, Addae and Lotspeich proposed that the liver was the site of glutamine production in the rat as well as in the dog.

The metabolism of selected amino acids by the kidney has been studied using the isolated perfused rat kidney (59). Using an artificial saline medium containing a single amino acid (one of alanine, glycine, leucine or aspartate) at 5 mM concentration, production of ammonia and glucose by the perfused kidney was measured. Pitts found increased ammonia production by the acidotic kidney from all four amino acids, and increased glucose production from alanine and aspartate. Using kidney cortex slices, increased activities of alanine aminotransferase and glutamate dehydrogenase were measured in the acidotic kidney. He thus suggested that alanine and aspartate may undergo transamination with  $\alpha$ -ketoglutarate to form glutamate before being deaminated. While his results are undoubtedly valid for the system studied they may have little



physiological significance. The presence of only one amino acid in such high concentrations will undoubtedly interrupt steady state metabolic levels. Experiments of this type are reminiscent of the loading experiments previously discussed.

The renal metabolism of amino acids was also investigated in fed and fasted pregnant sheep (13). There were significant outputs of glycine, serine, arginine and glutamine by the normal kidney and significant uptakes of alanine, aspartate, glutamate and citrulline. However, in 3-day fasted animals that were hypoglycemic, slightly acidotic, and excreting increased amounts of ammonia, glutamine was taken up and glutamate was put out by the kidney. Further studies (35) using both fed, starved and acidotic sheep showed that the acidotic sheep kidney also removes glutamine. The plasma glutamine level is decreased in acidosis and the authors report that "net peripheral release of glutamine tended to increase in acidotic and starved sheep", although no data were given.

Recently, work on the metabolism of glutamine in normal and acidotic humans has been expanded (23). In agreement with the results of Owen and Robinson (56), these authors find that glutamine extraction by the normal kidney amply accounts for the ammonia produced by the kidney. In acidosis, glutamine extraction increases, but accounts for only 80% of the total ammonia production; the renal extraction of glutamate does not account for the balance. These authors find that the human forearm normally produces glutamine, and this production increases during acidosis. The liver, however, does not produce glutamine but releases

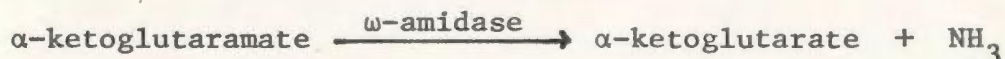
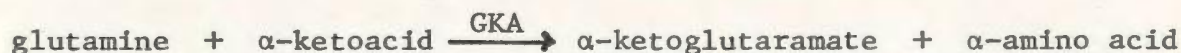


glutamate into the circulation.

## B. Pathways of Ammonia Production

The transfer of amino groups to  $\alpha$ -keto acids by transaminase enzymes has been mentioned briefly. The extent of contribution of this type of reaction to the production of urinary ammonia has not been established.

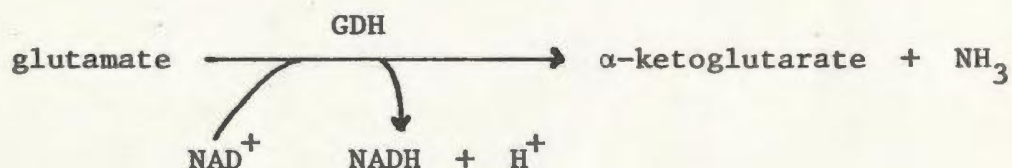
There are two main pathways known for the breakdown of glutamine; namely the glutaminase I and glutaminase II pathways. The glutaminase II pathway is catalyzed by the sequential actions of two enzymes glutamine-ketoacid aminotransferase (GKA) and  $\omega$ -amidase:



This system does not involve a true glutaminase reaction since the term glutaminase refers to an enzyme which directly converts glutamine +  $\text{H}_2\text{O}$  to glutamate +  $\text{NH}_3$ .

The glutaminase I pathway has been shown to be quantitatively the most important pathway of glutamine deamination (34). Glutaminase I has been characterized into phosphate dependent glutaminase (PDG) and phosphate independent glutaminase (PIG) both of which catalyze the breakdown of glutamine directly to glutamate and ammonia. Of these two isoenzymes, PDG is quantitatively the most important and has been localized in the mitochondrial matrix (42). PIG has been localized in the brush border of the kidney cortex cells (43). It has been shown (21, 75) not to be a true glutaminase but part of the  $\gamma$ -glutamyl transpeptidase system.

Glutamate is the end product of the glutaminase I pathway and also of the glutaminase II pathway when  $\alpha$ -ketoglutarate is the main  $\alpha$ -keto acid involved. Glutamate formed by glutamine deamidation may serve as an important source of urinary ammonia by the action of glutamate dehydrogenase (GDH), thus:



### III. RENAL LACTATE METABOLISM

Leal-Pinto et al. (46) have studied the renal metabolism of lactate in the alkalotic and acidotic dog. By infusing  $^{14}\text{C}$ -lactate, they have determined that, in alkalosis, the contribution of lactate to production of  $\text{CO}_2$  is twice as high as in acidosis, although the total production of  $\text{CO}_2$  does not differ in the two acid-base states. More lactate was extracted from the renal arterial blood of the dog in alkalosis than in acidosis. This work, together with work done previously on the renal metabolism of glutamine carbon (63) suggests that lactate metabolism of the normal kidney is reduced during acidosis to allow the metabolism of the glutamine carbon which remains after ammonia production.

Work has also been done on the effect of acidosis on the metabolism of lactate by the perfused rat kidney (88). Lactate removal by kidneys of normal rats was stimulated by a reduction in perfusate pH to 7.1-7.2

but no lower. However, kidneys from acutely acidotic rats (4-6 hours after oral  $\text{NH}_4\text{Cl}$  administration) showed no increased lactate removal compared to kidneys from normal rats. From such a study as this, however, where rats were made acidotic for such a short time, any changes due to acidosis may not have had time to be established. As well, such in vitro experiments have questionable physiological validity when concentrations of substrates are 5-10 times higher than physiological levels. The effect of acidosis on lactate removal by the rat kidney is therefore still open to further investigation.

#### IV. AMINO ACID TRANSPORT IN WHOLE BLOOD AND PLASMA

Measurements of the transport and turnover of amino acids in the body are important in a study of the control of acid-base balance. Methods used to detect changes in amino acid levels effected by an organ (for example, measurements of arterio-venous differences) involve the measurement of amino acid levels in the blood perfusing that organ. The question arises as to whether whole blood or plasma should be used in such measurements; the latter being preferred, if possible, due to the ease of handling of plasma samples and high glutathione content of red cells which makes the determination of some amino acids in whole blood rather difficult. The following discussion will reveal, however, that whole blood measurements should be made when studying interorgan transport of amino acids.

##### (A) Experiments in vitro.

The work up to the early 1960's on the in vitro transport of amino



acids between erythrocytes and plasma has been summarized by Winter and Christensen (84). Using human erythrocytes these authors tried to characterize the effect of the structure of a neutral amino acid on its rate of transport across the erythrocyte membrane. They proposed three mechanisms for neutral amino acid uptake: (a) a mediated transport of neutral amino acids, especially those having long side chains, (b) a transporter of alanine and glycine that is of low capacity allowing slow transport of these amino acids and (c) a non-saturable component limited to amino acids with large hydrocarbon side chains. The charged amino acids studied such as glutamic acid and aspartic acid were taken up very slowly by the red cells. No data are reported for the transport of glutamine across the erythrocyte membrane. Similar work on the human erythrocyte has been reported (15). Uptakes of labelled glycine, tryptophan, lysine, phenylalanine and glutamic acid were very slow; equilibration of amino acids between erythrocyte and suspending medium was still not obtained after 4 hours.

Amino acid transport in sheep erythrocytes in vitro has also been investigated (87). These workers have studied the effects of different amino acids on L-alanine transport and have proposed that both the neutral and dibasic amino acids share a common transporter. 50 mM glutamine had no effect on L-alanine transport suggesting that glutamine is not transported by the same carrier in sheep erythrocytes.

(B) Experiments in vivo.

A number of papers have appeared in the literature concerning the transport of amino acids across the erythrocyte membrane in vivo. Pitts

et al. (61) reported arterio-venous differences across the dog kidney in acidosis and alkalosis using both whole blood and plasma. However, the results that were reported represent only one set of measurements made on a single dog making it impossible to draw statistically valid conclusions from these data. However, the arterio-venous difference ( $\mu\text{moles/ml}$ ) obtained for glutamine on plasma was approximately twice that obtained using whole blood, indicating that the red blood cell glutamine content is probably not available to the kidney. Later work by Elwyn (26, 28) on the dog demonstrated that differences exist in amino acid levels between plasma and whole blood in vivo, which are not altered by incubation of the blood withdrawn from the animal. This difference is illustrated by calculation of the distribution ratio which is the ratio of the blood cell to plasma levels. To represent equal distribution between cells and plasma this ratio would be 1.0 for the neutral amino acids and 0.7 and 1.4 for the acidic and basic amino acids respectively due to Gibbs-Donnan effects. However, the distribution ratios for most amino acids are always greater than 1 and also differ depending upon from which blood vessel the blood sample was taken. This is due in part to the large changes which occur in plasma amino acid levels and in some cases (e.g., glutamic acid) to opposite changes in the red blood cell levels. This indicates that in vivo transport of amino acids across the erythrocyte membrane can occur as the blood perfuses an organ. Changes also occur in red blood cell amino acid levels as a function of time, for which there are no corresponding changes in plasma. In his later report (27) Elwyn remarked on the rapid transfer of amino acids into and out of the



red blood cell that occurs as the blood passes through a capillary bed, indicating a rapid increase in the rate of transport of amino acids in vivo across the erythrocyte membrane into plasma. The implication from this is that some special mechanism for amino acid transport occurs in vivo, e.g., direct transfer of amino acids between erythrocytes and tissues. He suggests that the movement of amino acids between erythrocytes and plasma may be affected by hormones.

Studies on amino acid transport by human erythrocytes in vivo were carried out by Aoki et al. (8). These investigators studied the effect of insulin on glutamate uptake by the human forearm. They found that using plasma to measure arterio-venous differences, no increase in uptake of glutamate by muscle could be measured after infusion of insulin. If measurements were made using whole blood, however, a significant uptake of glutamate could be measured after insulin treatment. In addition, in normal man, the arterio-venous difference for glutamate across the forearm using plasma was found to be much greater than that measured using whole blood. Thus, in the normal case, glutamate moves into the red blood cell and muscle cell from the plasma, while in the presence of insulin, glutamate moves out of the red blood cell and plasma and into the muscle cells. It is therefore possible that hormones such as insulin may regulate amino acid movements across the erythrocyte membrane, although the mechanism for this has not been postulated.

Shortly afterwards, Aoki et al. (7) studied the plasma versus whole blood arterio-venous differences for all amino acids across the human forearm. There was an overall release of amino acids from muscle,



especially of glutamine, alanine, glycine, proline, threonine and lysine. The total release of amino acids measured using plasma (385  $\mu$ moles/l. plasma) was greater than the total amino acid release from whole blood (237  $\mu$ moles/l. blood) by a fraction which corresponded to the blood cell volume (hematocrit), indicating minimal blood cell:plasma interactions in the basal state. Following the ingestion of 200 g boiled beef, whole blood glutamine levels did not change, however, arterial blood cell glutamine levels increased. Thus, the blood cell/plasma glutamine ratio appears to be a function of the glutamine concentration in both compartments.

These investigators also found differences between arterial blood cell and plasma amino acid levels; higher taurine, aspartate, glutamate, glycine, ornithine and lower proline,  $\alpha$ -amino-n-butyrate, valine, isoleucine, leucine, phenylalanine, lysine and arginine levels were found in blood cells than in plasma. Work in this area was continued by Felig et al. (30). These investigators measured arterio-venous differences for all amino acids across the leg and splanchnic bed of humans using both whole blood and plasma. They found higher levels of taurine, threonine, serine, citrulline, glycine, aspartate and alanine in whole blood than in plasma. Significant exchange of amino acids between tissues via the blood cells was calculated to occur for alanine, threonine, serine, methionine, leucine, isoleucine, tyrosine, and citrulline, since arterio-venous differences using whole blood were greater than arterio-venous differences using plasma (both measured as  $\mu$ moles/l. of whole blood). Glutamine uptake by the G.I.T. (arterial-portal venous difference)

using whole blood was much lower than that using plasma, indicating an uptake of glutamine by the blood cells as the blood perfuses the gut. Alanine was found to be the most important amino acid exchanged between organs accounting for 35-40% of the total amino acid flux from peripheral tissues and G.I.T. to the liver. Transport via blood cells accounts for 22-32% of the total net movement of this amino acid. Windmueller and Spaeth (83) however have reported that preliminary experiments showed no equilibration of  $^{14}\text{C}$  glutamine between plasma and cells in a single pass through perfused intestine.

These studies make it clear that the blood cellular elements may be important carriers in the net flux of amino acids between tissues, although transport of amino acids across the blood cell membrane in vitro is very slow. The mechanisms involved in amino acid transport by erythrocytes have yet to be elucidated. It is important, however, that in studies of amino acid flux, measurements using both whole blood and plasma be made.

## V. INTERORGAN TRANSPORT OF AMINO ACIDS IMPORTANT IN NITROGEN METABOLISM

### (A) Changes in Levels of Amino Acids with Altering Acid-Base Status.

Changes in blood amino acid levels with changing acid base balance have been noted by some investigators. Shalhoub et al. (71) measured arterio-renal venous differences in the dog for 23 amino acids using plasma. They report higher plasma arterial levels of glutamate, alanine,



serine, glycine, proline, threonine, histidine and arginine in acidosis than in alkalosis. However, as pointed out previously, these shifts in concentration could reflect a movement of amino acids into and out of red blood cells. Addae and Lotspeich (2) report elevated or unchanged levels of arterial plasma glutamine in acidosis compared to normal dogs.

However, Owen and Robinson (56) note a fall in plasma glutamine during acidosis ( $P < .001$ ) from a value of  $65 \pm 7.5$   $\mu\text{moles}/100$  ml plasma in the normal to  $40 \pm 5$   $\mu\text{moles}/100$  ml plasma in the acidotic human. Although a general lowering of most amino acid levels was observed in arterial plasma from 9 day acidotic humans, glutamine was the only amino acid present in significantly decreased amounts. Felig *et al.* (29) have also reported a lowering of arterial plasma glutamine in diabetic patients [normal =  $0.554 \pm .033$  (S.E.)  $\mu\text{moles}/\text{ml}$ ; diabetic =  $0.424 \pm .037$  (S.E.)  $\mu\text{moles}/\text{ml}$ ]. Weiss and Preuss (80) report acidotic arterial plasma glutamine levels of  $0.68 \pm 0.02$  (S.E.)  $\mu\text{moles}/\text{ml}$  for the rat; no normal values were reported. Welbourne (81) reports no significant difference from the normal levels of arterial plasma glutamine for rats made acidotic by replacing their drinking water with 1.5%  $\text{NH}_4\text{Cl}$  for 1 week [normal =  $0.592 \pm 0.045$  (S.E.)  $\mu\text{moles}/\text{ml}$ ;  $\text{NH}_4\text{Cl}$  acidotic =  $0.610 \pm 0.017$  (S.E.)  $\mu\text{moles}/\text{ml}$ ]. However, rats made acidotic by intraperitoneal injection of acetazolamide (an inhibitor of carbonic anhydrase) for 7 days showed a significant decrease in arterial plasma glutamine ( $0.405 \pm .033$   $\mu\text{moles}/\text{ml}$ ). Lund and Watford (51) presented data in a recent symposium that showed a significant decrease ( $P < .05$ ) in whole blood glutamine levels during chronic acidosis in rats. Heitman and Bergmann



(35) also have recently reported that during acidosis and fasting plasma glutamine levels decrease in the sheep.

(B) Measurements of Amino Acid Exchange In Vivo.

Numerous studies have been made on the exchange of amino acids between organ systems. Hills et al. (38) measured an output of glutamine by the hindlimb of mongrel dogs, greyhounds and spider monkeys. Infusion of ammonium salts was found to increase glutamine output, although ammonia uptake before infusion was not sufficient to account for the amide nitrogen of the released glutamine. There was also a significant glutamine output by the brain (arterial-external jugular venous difference). The G.I.T. (arterial-portal venous differences) as well as the kidneys remove glutamine. Animals studied were, however, starved for 32 hr and glutamine was determined on plasma samples. These results contrast with the findings of Addae and Lotspeich (2) who studied glutamine metabolism in dogs in acute sulfuric acid acidosis. These workers find no glutamine output by the brain or hindleg of the normal dog; in the acutely acidotic dog, they find glutamine uptake by the brain. They also find that glutamine is produced by the liver, and that production increases in acidosis. They suggest that the liver is the major site of glutamine production in the dog.

Aikawa et al. (4), Matsutaka et al. (53) and Yamamoto et al. (86) have studied the interorgan transport of some amino acids in fed and starved rats. They found that alanine and glutamine accumulated to the greatest extent in the plasma after the G.I.T., liver, and kidneys were removed from the circulation. Others workers (16) have shown that

alanine release can be decreased by stimulation of pyruvate dehydrogenase with dichloroacetate and by inhibition of alanine aminotransferase with cycloserine. Glutamine release was decreased by inhibition of glutamine synthetase with methionine sulfoximine.

Arterio-venous difference measurements made using plasma (4, 53, 86) show that, in the normal rat alanine and glycine are released by the muscle, G.I.T. and kidneys. Glutamine is removed by the G.I.T. and the kidneys and put out by the splanchnic bed (liver + G.I.T.). The liver removes alanine and glycine from the blood. Smaller transfers of lysine from the muscle to the liver and of serine from the kidneys to the G.I.T. were also measured.

Glutamine is produced by the hindquarter of the starved rat (67). A large release of alanine and glutamine by muscle has been reported in postabsorptive and starved man (30, 52) and in normal and diabetic man (29). Outputs of these two amino acids are reported at 1.5 to 2 times their proportions in muscle. All workers have indicated that alanine is removed primarily by the splanchnic bed, while glutamine is removed by both the splanchnic bed and kidneys. Some authors (29) report also that transfer of glutamate occurs from the splanchnic bed to muscle. It has been pointed out (65) that in the starved rat glutamine release from muscle is unchanged while alanine release increases tremendously. In rats where blood glucose has been lowered by injection of phloridzin (a substance which inhibits glucose absorption by the kidney), alanine release by muscle and extraction by the liver is increased. Glutamine has also been reported to be released by the brain in normal fed rats (50).



It thus appears, from the above studies in vivo, that alanine and glutamine are important nitrogen carriers in the blood. Alanine is apparently synthesized in the muscle by the transfer of nitrogen to pyruvate and is important in the transport of nitrogen to the splanchnic bed and as a gluconeogenic precursor. Glutamine is released by muscle and is important as a source of nitrogen in kidney metabolism. Since alanine and glutamine are released in such large amounts while the acidic and branched-chain amino acids are released in such small amounts, it appears that these latter amino acids provide the nitrogen for glutamine and alanine synthesis.

(C) Measurements of Amino Acid Exchange In Vitro.

Work carried out in vitro on amino acid metabolism has also demonstrated the importance of alanine and glutamine as nitrogen carriers. These amino acids accounted for 50% of the total amino acids released in the perfused rat hindquarter (66, 67). Alanine release, but not glutamine release, was increased in perfused hindquarter from starved rats. Glutamine release and tissue glutamate levels were increased by addition of leucine to the perfusate. Addition of  $\text{NH}_4\text{Cl}$  to the perfusate also increased glutamine release. Similar results were obtained by other authors using an intact epitrochlearis muscle preparation (31, 32). They have also shown that other amino acids besides leucine can supply both the carbon and nitrogen for glutamine and alanine synthesis. It is thus apparent that, in the intact animal, glutamine and alanine are synthesized from other amino acids released by tissue catabolism.

Glutamine metabolism by the G.I.T. has been studied in vitro using



perfused rat intestine (82, 83). Glutamine was removed from the perfusate by preparations from normal and germ-free rats. Although 20% of the total plasma glutamine is removed in each pass of the blood through the intestine, a stable lower level of glutamine is normally maintained. This level is decreased by the addition of methionine sulfoximine, indicating that the intestine also synthesizes glutamine. The metabolism of glutamine occurs mainly in the small intestine mucosa and accounts for 30% of the total  $\text{CO}_2$  produced by the preparation. The nitrogen from glutamine can be accounted for in germ-free rats by the ammonia, citrulline, alanine and proline released into the circulation. Glutamine removed from the lumen is metabolized in a similar fashion.

Glutamine metabolism has also been studied in the isolated perfused rat liver. Net output of glutamine by the liver was greatest at pH 7.15 due to a drastic decrease in glutamine catabolism (49). Other workers (76) have shown that at pH 6.9, glutamine production from 100 mM  $\text{NH}_4^+$  and 10 mM alanine was increased. The physiological relevance of these studies is questionable, however, not only because of the high substrate levels involved, but because it has been shown (57) that blood pH values return to normal five days after the start of  $\text{NH}_4\text{Cl}$  administration. Thus, changes in blood pH may play a role in the stimulation of hepatic glutamine production only in acute acidosis.

A net uptake of glutamine has also been measured in the perfused rat liver (50) when glutamine levels in the perfusate were greater than 1 mM. These workers also found that ligation of both the portal vein and hepatic arteries in the intact rat, thus eliminating the

at pH 7.1 (36). In addition, kidney cortex slices from acidotic rats utilize more glutamine than do slices from normal rats (5). These workers have also found that preincubation of normal slices with acidotic plasma caused increased glutamine utilization by these slices, and have suggested that there is a factor present in the plasma which causes increased ammonia production. Lotspeich (47) has reported that an increase in kidney size occurs during acidosis in the dog. This may be important in increasing the capacity of the animal to excrete excess acid during sustained metabolic acidosis.

## VI. MEASUREMENTS OF THE TURNOVER RATE OF GLUTAMINE

### A. Theory

Investigators have followed two major approaches in using labelled substances in the measurement of turnover rates of metabolites. These involve the administration of the labelled compound as either a single injection or as a continuous infusion at a constant rate. The evolution of these techniques and their relative strengths and weaknesses have been reviewed by Hetenyi and Norwich (37).

Zilversmit et al. (89, 90) derived a relationship whereby the turnover rate of a substance in a tissue (i.e., the amount of a substance that is turned over by that tissue per unit of time) could be calculated following a single injection of that substance which has been labelled in some way. Turnover refers to the removal of a substance, and may be due to transport of the substance out of the body pool and/or breakdown of that substance with subsequent release of label. Simplifying as-



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assumptions made in the derivation of formulae are that the substance is in a steady state (i.e., is at a constant concentration), having a constant rate of disappearance and appearance. Also, it is assumed that labelled molecules react as do unlabelled molecules. Thus the specific activity of that fraction of the total pool of the compound being broken down or leaving the body pool is equal to the specific activity of the total amount of the compound present in the body pool.

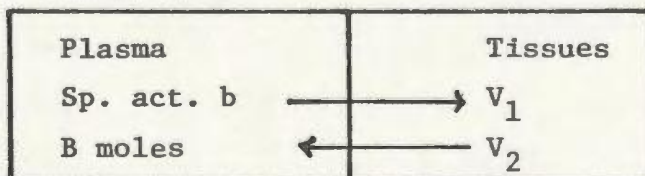
Thus, one can determine the turnover rate of a labelled substance in the plasma by the following:

Let  $B$  = the total amount of the substance present in the plasma,  
i.e., the pool size (moles)

$b$  = the specific activity of the substance in the plasma (curies/  
mole)

$V_1$  = the rate of disappearance of the substance from the plasma  
(moles/time)

Using the following simple model consisting of the two compartments, plasma and all tissues which it perfuses,  $V_1$  is the net flux of the substance out of the plasma



The rate of change of radioactivity in the plasma is given as:

$$\frac{d(bB)}{dt} = -V_1 b$$

$$\frac{db}{b} = \frac{-V_1}{B} dt$$

$$\text{on integration, } \ln b = \frac{-V_1}{B} t + \text{constant} \quad (1)$$

A plot of  $\ln b$  versus  $t$  will thus have a slope of  $-V_1/B$ . The net flux out of the plasma  $V_1$  can thus be calculated if the pool size ( $B$ ) is known. In practice this can be calculated if the amount of radioactivity at time zero  $(Bb)_0$  injected into the plasma is accurately known. A linear relationship is predicted between  $\ln b$  and  $t$  by equation 1 and this is usually obtained after an initial multi-exponential decrease which is presumably due to mixing of the label throughout the body pool (Fig. 3). By extrapolation of the  $\ln b$  vs  $t$  line back to zero time, one can calculate the "zero time specific activity  $(b_0)$ ", i.e., the specific activity the substance in the blood would be at zero time if instantaneous equilibration of label throughout the body pool occurred. Assuming the pool size did not change after the equilibration period, the calculated pool size at time zero  $(B_0)$  is determined from:

$$B_0 = \frac{(Bb)_0}{b_0}$$

This simple derivation does, however, have several drawbacks. First of all, no consideration is made for back-flow of labelled substances into the plasma or for resynthesis of labelled substances from labelled metabolites. However, if a straight line is obtained for the  $\ln b$  versus  $t$  plot it means that either that no re-entry of label into plasma has occurred or that re-entry of label has occurred at a constant rate. Thus, one obtains for the net flux a combination of  $V_1$  and  $V_2$ , that is, a combination of the fluxes out of and back into the plasma. It is also apparent that the flux  $V_1$  is in fact a combination of an individual

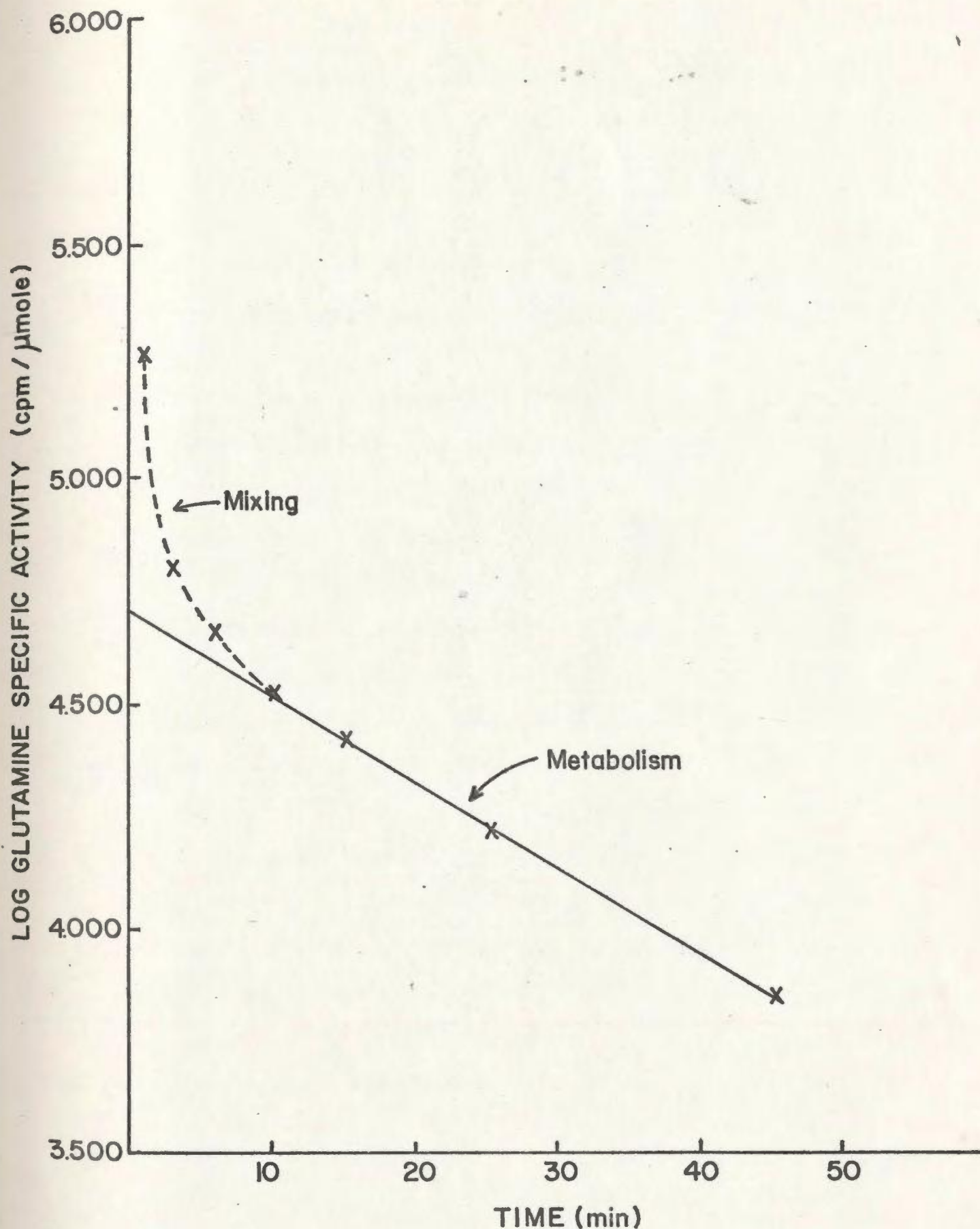


FIG. 3. Single Injection Method for the Measurement of Glutamine Turnover.

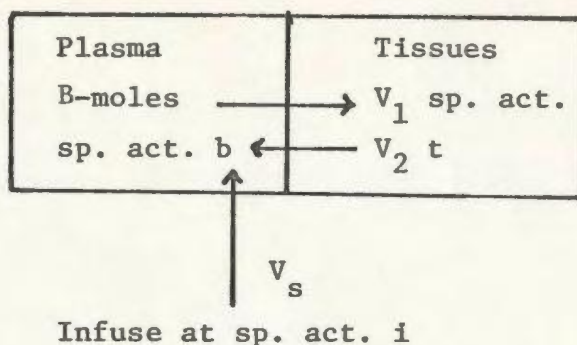


flux values for the substance into the various tissues (i.e.,  $\sum_{i=1}^n V_{1i}$ ). It is very likely that the flux rate of a substance out of plasma into a tissue varies in different tissues.

The measurement of the turnover rate ( $V_1$ ) was undertaken in this study in an attempt to detect changes in glutamine metabolism between normal and acidotic rats; in particular, I hoped to detect changes in the rate of synthesis and/or degradation of glutamine in extrarenal tissues of the acidotic rat which would provide the extra glutamine required by the acidotic kidney for urinary ammonia production. However, the change in the fractional part of  $V_1$  due to renal metabolism must be a significant fraction of the total  $V_1$  for glutamine in order to detect any changes in  $V_1$  with altered acid-base status.

The turnover rate of a substance may also be determined by a method based on the constant infusion of labelled substances. In this method the labelled substance is administered as a continuous infusion at a constant rate until the label has equilibrated throughout the body pool. After this point the label remains at a constant specific activity, i.e., at a plateau level. The rate of removal of label is then equal to the rate of infusion of label and the flux may be easily determined.

In this derivation, the simple two compartment model is considered consisting of the plasma and all tissues which it perfuses. Labelled substance is infused into the plasma at a rate of  $V_g$  (moles/time) and specific activity  $i$  (curies/mole).



The rate of change of label in the plasma is given by:

$$\frac{d(Bb)}{dt} = V_s i + V_2 t - V_1 b$$

When the label in the plasma is at a constant specific activity (i.e., at plateau) then,

$$\frac{d(Bb)}{dt} = V_s i + V_2 t - V_1 b = 0$$

assuming no backflow of label into the plasma (i.e.,  $V_2 = 0$ )

$$V_s i = V_1 b$$

$$\text{and } V_1 = \frac{V_s i}{b}$$

$V_s i$  is the rate of infusion of label (curies/time). As in most tracer experiments, the mass of the labelled substance infused is assumed to be negligible in comparison to the amount of that substance in the body pool. No selective treatment of labelled molecules and no re-synthesis of labelled molecules are assumed. The continuous infusion technique does not require accurately timed samples as does the single injection technique and it may thus be technically advantageous.

The flux measured by both these techniques is a combination of component

fluxes into different tissues as well as the rate of degradation of labelled compound in these tissues. The pool size may thus be greater than the plasma and will include the amount of the substance in all tissues across which the label quickly equilibrates. The use of these techniques assumes that no recirculation of label (i.e., resynthesis of labelled glutamine) has occurred. This may have occurred in some tissues, notably the kidney which not only removes glutamine from the blood but contains an active glutamine synthetase. I hoped to minimize the resynthesis of labelled glutamine by using  $^{14}\text{C}$ -glutamine which was labelled in the 1-carbon position. Thus,  $^{14}\text{C}$  label is released from glutamine after the breakdown of  $\alpha$ -ketoglutarate to succinyl CoA (Fig. 4).

#### B. Measurement of Turnover Rates Reported in the Literature

The turnover rate of glutamine and other amino acids has been measured in intact animals and in various preparations in vitro. Duda and Handler (25) measured the turnover of glutamine in liver after the injection of glutamine-amide  $^{15}\text{N}$  into the blood. They obtained a value of 2.7 hours for the turnover time (i.e., the time it takes for the entire pool to turnover) which when taken with a measured total body pool of 1.25 mmoles, gave a value of  $463 \mu\text{moles/hr}/300\text{--}400 \text{ g rat} = 120\text{--}150 \mu\text{moles/hr}/100 \text{ g}$  for the turnover rate of body glutamine. These values for turnover were obtained from the limiting slope of the log glutamine specific activity versus time curve as discussed in the previous section. In the calculation of the turnover rate given above it



# PATHWAYS OF GLUTAMINE METABOLISM

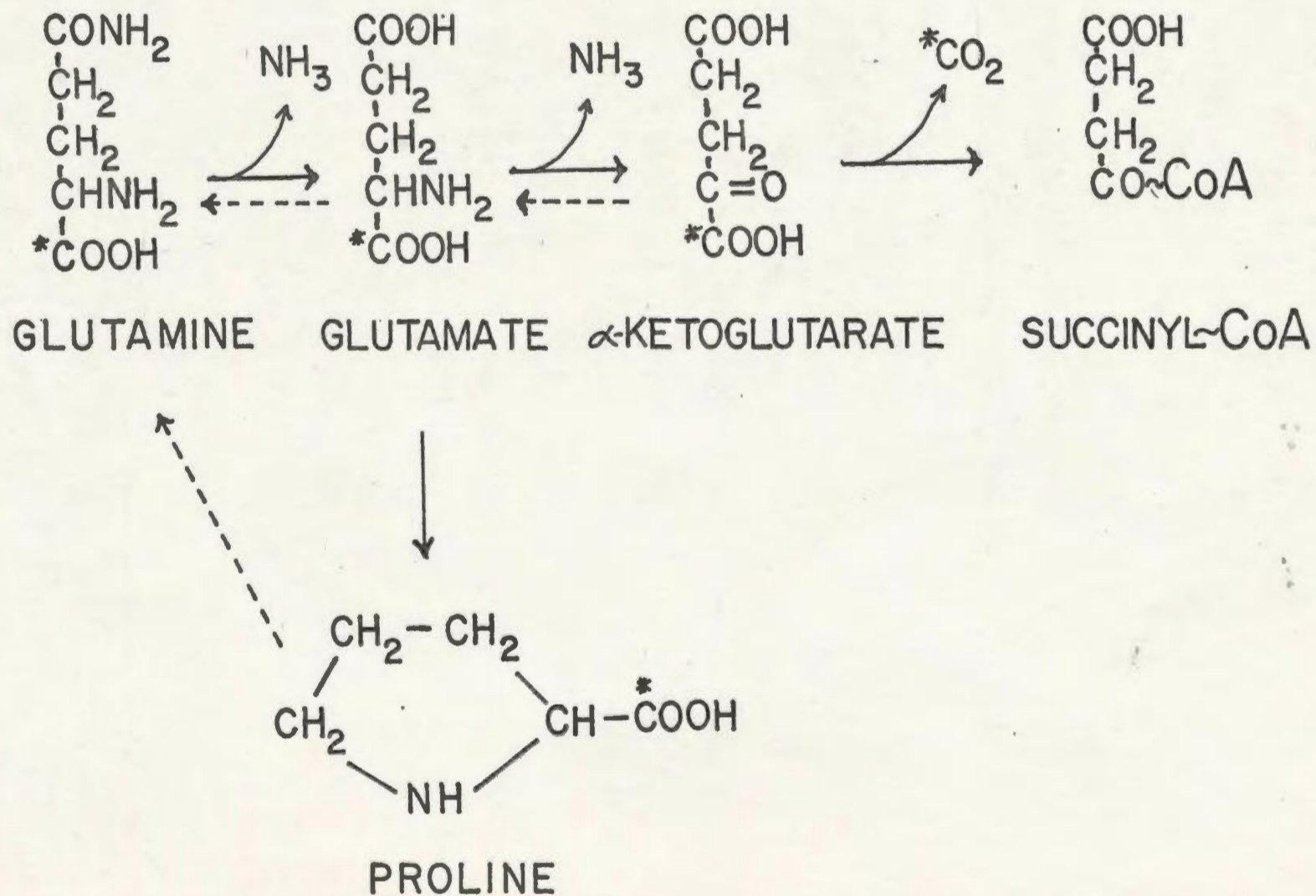


FIG. 4. Pathways of Glutamine Metabolism.

is assumed that the  $^{15}\text{N}$  glutamine administered equilibrates with the total body glutamine pool. These authors also measured the rate of glutamine synthesis from  $^{15}\text{NH}_3$ ; the administration of  $^{15}\text{N}$  ammonium lactate resulted in 80% of the label appearing in amide of glutamine. Thus, if  $\text{NH}_3$  liberated by glutamine breakdown was metabolized in the same manner as the exogenous ammonia load, 80% of the ammonia was re-synthesized into glutamine. Thus, the real turnover time is about 1/5 of the apparent value or approximately 0.54 hours. Thus, the turnover rate given by this method may be as high as 570 to 770  $\mu\text{moles/hr/100 g}$  body wt. However, this calculation assumes that the metabolism of endogenous ammonia in the liver occurs by the same route as an injected load of ammonium lactate.

The turnover rate of glutamine has been measured in sheep (35) by constant infusion of  $\text{U-}^{14}\text{C}$  glutamine. These authors have determined values of  $12.2 \pm 0.7$ ,  $13.1 \pm 0.3$  and  $12.0 \pm 0.9$   $\text{mmoles/hr}$  for fed,  $\text{NH}_4\text{Cl}$  acidotic and three day starved sheep. There appears to be little difference in glutamine turnover rates in these different states.

Turnover rates for other amino acids have been measured by a number of authors. Waterlow and Stephen (79) have reported values for lysine turnover in the rat of approximately 180  $\mu\text{moles/hr/100 g}$  body wt. They have compared this to values of 43, 18, 43 and 66  $\mu\text{moles/hr/100 g}$  obtained using valine, isoleucine, leucine and glycine respectively by other workers. Thus, most amino acids probably turnover at significantly lower rates than glutamine. Glutamine turnover has also been determined in several in vitro preparations. Windmueller and Spaeth (82) have

measured the rate of glutamine utilization by the small intestine to be 179 nmoles/min/g tissue. Thus, intestine utilizes 75  $\mu$ moles/hr/280 g rat or 27  $\mu$ moles/hr/100 g body wt. Using the turnover rate of Duda and Handler (25) of 120-150  $\mu$ moles/hr/100 g rat, 18-23% of the total rate of glutamine turnover is due to the utilization of glutamine by the small intestine.



## MATERIALS AND METHODS

## I. MATERIALS

A. Reagents

Chemicals used were of analytical grade or equivalent and were obtained from either J.T. Baker Chemical Co., Phillipsburg, N.J. or Fisher Scientific Co., Fair Lawn, N.J. All enzymes were obtained from Sigma Chemical Co., St. Louis, Missouri.

B. Radioisotopes

L-U<sup>14</sup>C-glutamine, <sup>14</sup>C-carboxy-dextran and <sup>3</sup>H<sub>2</sub>O were obtained from New England Nuclear. L-1<sup>14</sup>C-glutamic acid was obtained from Amersham Searle Corporation. L-1<sup>14</sup>C-glutamine could not be obtained from any supplier and was thus synthesized as described later. The L-U<sup>14</sup>C glutamine was separated from possible breakdown products (glutamate and 2-pyrrolidone-5-carboxylate) by the procedure of Adam and Simpson (1). Radiochemical purity was confirmed as described later.

C. Animals

Male Sprague-Dawley rats weighing 300-400 g used in all experiments, were obtained from Canadian Breeding Farms and Laboratories, St. Constant, La Prairie, Quebec. They were kept under continuous light conditions with free access to Purina Rat Chow (Ralston Purina of Canada, Ltd., Don Mills, Ontario) containing a minimum of 22.0% crude protein, a minimum of 4.0% crude fat, a maximum of 5.0% crude fiber as well as all necessary vitamins and mineral supplements. Water was given ad libitum

unless acidosis was to be induced when 1.5%  $\text{NH}_4\text{Cl}$  was substituted for the drinking water for 7 to 10 days. After receipt of each shipment of animals, they were conditioned in our own animal room, and given unlimited food and water for at least one week prior to use.

Previous experiments on the induction of acidosis in the rat (72) showed that when rats weighing less than 250 g were given 1.5%  $\text{NH}_4\text{Cl}$ , they ate poorly and lost weight, as well as having a lowered liver glycogen level. Therefore, in all experiments described here, rats weighing more than 250 g were used. Prior to measurements of arterio-venous differences and in measurements of turnover rates, errors due to gross uptakes of amino acids from the G.I.T. were minimized by withholding food for four hours before the beginning of the experiment. Examination of the gut by dissection showed the gut to be almost empty when food was withheld for this length of time.

## II. MEASUREMENT OF ARTERIO-VENOUS DIFFERENCES

### A. Anaesthesia

In early experiments, rats were anaesthetized by the intraperitoneal injection of sodium pentobarbital (6.5 mg/100 g body wt). However, when longer-acting anaesthesia was required, as in the experiments measuring the turnover rate of glutamine, inactin (0.1 ml of a 100 mg/ml solution in isotonic saline/100 g body wt) was injected intraperitoneally. (Inactin is the sodium salt of ethyl-(1 methyl-propyl)-malonyl-thiourea and was obtained from Henley and Co. Inc., New York, N.Y.) Small

additional doses of anaesthetic were administered if needed.

Preliminary attempts were made at measuring the blood pressure of normal and acidotic rats using a tail cuff before and after inactin anaesthesia. Measurements on two normal animals showed no lowering of the blood pressure under inactin anaesthesia. Work by other investigators who used inactin also showed a stable and normal blood pressure under inactin anaesthesia (55, 69, 74, 85). Presumably then, no detrimental effects on glomerular filtration rates were caused by inactin.

Arterio-venous differences for glutamine across the kidney were also measured on both normal and acidotic rats under both sodium pentobarbital and inactin anaesthesia. Results showed that equivalent amounts of glutamine were removed by the acidotic rat kidney when either anaesthetic was used.

#### B. Blood Sampling

Blood samples were taken, in different experiments, from the right external jugular vein, right femoral vein, hepatic portal vein and right renal vein using 26 gauge x 1/2" needles, from the hepatic vein using a 25 gauge x 1 1/2" needle bent to go over the liver and from the abdominal aorta using a 21 gauge x 1 1/2" short bevel needle. 1 ml or 5 ml syringes were attached and heparinized, if needed, by rinsing with a heparin solution (10,000 units/ml) obtained from M.T.C.

Pharmaceuticals. The jugular vein was exposed by cutting away a small section of skin (1/2" x 1/2") with Mayo scissors and clearing by blunt dissection. The needle was placed in the vein so that the venous blood



was sampled coming from the direction of the brain. The abdominal vessels were exposed by a midline incision along the linea alba and traction was applied, using wire hooks connected by rubber bands, to separate the edges of the incision. The vessels were cleared by blunt dissection and the appropriate needle inserted against the direction of blood flow, except the portal venous samples, which were withdrawn in the opposite direction due to technical considerations. In all experiments where a particular artery and vein were to be sampled, the venous sample was always withdrawn first, the needle left in place and the arterial sample then quickly taken. When the hepatic venous blood was to be sampled, a loose tie was first placed around the vena cava directly above the right renal vein. After all other venous samples were taken, the tie around the vena cava was tightened, the liver gently pulled away from the diaphragm to expose the hepatic vein and a bent needle was inserted to sample the hepatic venous blood.

When plasma was to be prepared, the blood was taken into heparinized syringes and, when sufficient blood was taken, the needle was removed and the blood was transferred to Eppendorf micro test tubes. These were centrifuged at  $12,000 \times g$  for 2 min in an Eppendorf micro centrifuge (Brinkman Instruments Inc., Toronto) to separate the plasma from the blood cellular elements.

#### C. Determination of Hematocrits.

Hematocrits were determined on arterial blood samples from normal and acidotic rats. Blood samples were taken up into heparinized capillary tubes (1.2 mm x 75 mm), sealed, and centrifuged at top speed ( $5,000 \times g$ )

in a clinical desktop centrifuge (IEC Model CL) for 5 min. Hematocrits were then immediately estimated using a microhematocrit tube reader (Sherwood Medical Industries Inc., Bridgeton, Mo.). Six different capillary tubes were filled with any particular sample and hematocrit values obtained were averaged.

D. Preparation of Samples for Analysis of Amino Acids and Lactate

Whole blood samples were deproteinized using an equal volume of 6%  $\text{HClO}_4$  while plasma samples were added to one-half volume of 12%  $\text{HClO}_4$ . These were kept on ice for at least 15 min or frozen overnight. Clear extracts were obtained by centrifugation.

In earlier analyses which were performed using sodium citrate buffers, clear perchloric acid extracts were added to an equal volume of sodium citrate buffer (0.2 N in Na) pH 2.2 and sufficient NaOH was added to return the pH to 2-3. Amino acid analyses were then performed on these extracts. However, these techniques did not allow the separation of glutamine from asparagine. Later methods were developed using lithium citrate buffers which gave good separation of glutamine from asparagine. When these methods were used, perchloric acid extracts were added to an equal volume of lithium citrate buffer (0.3 N in Li, 0.1 N in citrate) pH 2.2. Sufficient LiOH was then added to return the pH to 2-3.

Perchloric acid extracts to be assayed for lactate were adjusted to approximately pH 7 with KOH and the  $\text{KClO}_4$  produced was removed by centrifugation.

#### E. Determination of Amino Acids and of Lactate

In earlier experiments, amino acids were measured on a Beckman Model 121 automatic amino acid analyzer. Two different types of analysis were performed, (a) the analysis of Benson and Patterson (12) to determine the full spectrum of amino acids without resolution of glutamine from asparagine, and (b) the accelerated analysis of Benson et al. (11) to permit resolution of glutamine and asparagine. The following adaptations of the published methods were employed. In the analysis of acidic and neutral amino acids a 53 x 0.9 cm column was employed, the sodium citrate buffers were changed at 185 min from pH 3.16 (0.20 M in Na) to pH 4.25 (0.20 M in Na), the temperature change time was 140 min and the total analysis time was 400 min. In the analysis of basic amino acids the first sodium citrate buffer was at pH 4.25 (0.38 M in Na), the second buffer was at pH 5.36 (0.35 M in Na), the initial temperature was 30° C and the total analysis time was 300 min. In the accelerated method for glutamine and asparagine, HP-AN-90 resin (Hamilton Co., Whittier, California) was employed in a 36 x 0.90 cm column. The total analysis time was only 95 min since 10 min after the elution of glutamine the run was ended. The column was then regenerated with 0.3 M LiOH, and equilibrated with the starting buffer. Using these methods it was possible to measure the amino acids without interference from glutathione.

In later experiments, amino acid analyses were performed on the Beckman Model 121 amino acid analyzer by a single column lithium method which employed a DC-6A resin and the Durrum Pico Buffer System IV



(Durrum Chemical Corporation, Palo Alto, California). Some analyses were also performed on a Beckman Model 121 M amino acid analyzer as described in Beckman Bulletin No. 121 M - TB005 (July, 1976). Use of this machine allowed the analysis of micro sample volumes and thus permitted the taking of numerous blood samples from the same rat without seriously depleting its blood volume.

Lactate was measured in deproteinized blood samples, previously adjusted to pH 7, by the method of Lowry and Passonneau (48).

### III. MEASUREMENTS OF TURNOVER RATES OF GLUTAMINE

The turnover rate of L-1<sup>14</sup>C-glutamine was measured in the intact rat by two methods; (1) constant infusion and (2) single injection. The experimental techniques involved in both methods were similar. In both techniques, 1-<sup>14</sup>C-glutamine was administered via a jugular venous catheter and blood samples were withdrawn at timed intervals from a cannula implanted in the carotid artery. Radioactivity in 1-<sup>14</sup>C glutamine was then measured in these samples and the results used in the calculation of turnover rates.

#### A. Operative Procedure

Rats were weighed and anaesthetized by intraperitoneal injection of inactin (0.1 ml of 100 mg/ml solution in 0.9% NaCl/100 g body wt). The animal was then pinned to a wax board and the right external jugular vein was exposed by a small incision in the skin. The vein was then cleared

and loose ties placed. A catheter of PE 50 polyethylene tubing (I.D. .023"; O.D. .038") (Clay Adams), previously filled with heparinized saline (10 units/ml), was then inserted into the vein towards the heart to a distance of about 5 cm and then withdrawn slightly. Blood was drawn back into the catheter to ensure it was in the vein and ties then were tightened. The left carotid artery was next exposed and a catheter of PE 50 tubing implanted in a similar fashion and clamped with a forceps to prevent blood loss. Heparin (0.1 ml of 10,000 units/ml) was injected via the jugular venous catheter before the administration of label.

In the constant infusion experiments, 1-<sup>14</sup>C-glutamine in Krebs-Henseleit (4-5  $\mu$ Ci/ml) was infused via the jugular venous catheter using a constant infusion pump (B. Bawn Perfusor, Quigley Rochester Inc., Rochester, N.Y.). Following an initial priming dose of 0.7 ml (3-3.5  $\mu$ Ci), which was administered in about 5 seconds, the 1-<sup>14</sup>C glutamine was infused at a constant rate of about 1.9 ml/hr for 60-90 min. Samples were taken from the arterial cannula at known times for analysis of 1-<sup>14</sup>C glutamine radioactivity. Samples were also taken at the beginning and end of the infusion for the analysis of plasma glutamine levels. The withdrawal of samples was timed so that the volume of Krebs-Henseleit-<sup>14</sup>C glutamine infused would replace the blood volume taken. Throughout the infusion, the rectal temperature was monitored using a thermometer and the rat was heated by a tungsten lamp. The respiration rate was determined throughout and in some experiments, blood pH, pCO<sub>2</sub> and pO<sub>2</sub> were determined during the infusion. All measurements assured that the animal's condition was stable. (Rectal temp 35-37.5° C, respiration



rate 70-90 breaths/min,  $p\text{CO}_2$  40-50 mm Hg,  $p\text{O}_2$  70-90 mm Hg, pH 7.35-7.45).

The stability of the infusion pump was checked by pumping  $^{14}\text{C}$ -label at a constant setting into scintillation vials containing 15 ml Aquasol (New England Nuclear). In one experiment, twenty infusions of five minutes each were carried out and the  $^{14}\text{C}$ -label pumped averaged  $14,373 \pm 325$  cpm. At the end of each infusion, the pump was calibrated by three infusions of two minutes each into scintillation vials containing Aquasol. This enabled the measurement of dpm infused/hr, a value that was needed in the calculation of the turnover rate.

The single injection experiments were carried out using a similar technique, although an extremely accurate infusion pump is not necessary.  $1\text{-}^{14}\text{C}$ -glutamine label (6.4  $\mu\text{Ci}$ ) was administered as one bolus (0.6 ml) into the jugular venous catheter via a Y-connection made using two 23 gauge needles. This arrangement allowed the continuous infusion of Krebs-Henseleit bicarbonate throughout the course of the experiment. The infusion of Krebs-Henseleit bicarbonate was carried out at approximately 3.8 ml/hr and was timed so as to replace fluid lost in the withdrawal of blood samples from the arterial cannula.

#### B. Measurement of Specific Radioactivity in $1\text{-}^{14}\text{C}$ -Glutamine and $1\text{-}^{14}\text{C}$ -Glutamate

The accurate measurement of radioactivity in  $1\text{-}^{14}\text{C}$ -glutamine is necessary for the measurement of turnover rates. Previously published methods for the determination of radioactivity in  $^{14}\text{C}$ -glutamine and  $^{14}\text{C}$ -



glutamate involved in the use of paper chromatography (14), ion exchange chromatography (1) or the use of enzymes which require (a) the adjustment of pH between steps in the determination and (b) the use of more than one sample (20). All of these methods did not appear entirely satisfactory and thus a new method was developed which allowed the stepwise determination of  $^{14}\text{C}$ -label in the plasma bicarbonate, the 1-carbon of glutamic acid and the 1-carbon of glutamine on a single plasma sample.  $^{14}\text{C}$ -labelled bicarbonate was released as  $^{14}\text{CO}_2$  by incubation of the plasma sample at pH 5.0. Glutamic acid 1- $^{14}\text{C}$ -label and glutamine 1- $^{14}\text{C}$ -label were then removed by the successive additions of glutamate decarboxylase and glutaminase respectively. The  $^{14}\text{CO}_2$  produced in each step was trapped in center wells containing a strong base.

Advantages of this method included; (i) there was no necessity for the adjustment of pH between samples since both E. coli glutamate decarboxylase and E. coli glutaminase were active at pH 5.0, (ii) the radioactivity in both 1- $^{14}\text{C}$ -glutamine and 1- $^{14}\text{C}$ -glutamic acid was easily determined on a single blood plasma sample, (iii) high accuracy is obtained since only the actual plasma sample had to be accurately pipetted, (iv) determination of radioactivity in plasma  $^{14}\text{C}$ -bicarbonate, 1- $^{14}\text{C}$ -glutamine and 1- $^{14}\text{C}$ -glutamic acid could be routinely performed in 2 to 2 1/2 hours. The principal limitation of the method was the requirement of a preparation of glutamate decarboxylase which neither decarboxylates substances other than glutamate nor contains glutaminase activity. Suitable tests to determine the specificity of the glutamate decarboxylase preparation should therefore be made.

Glutamic acid and glutamine levels in plasma were determined as described and used for the calculation of specific activities.

### C. Preparation of L-1<sup>14</sup>C-Glutamine

L-1<sup>14</sup>C-glutamine was prepared using 25 units of glutamine synthetase (L-glutamate:ammonia ligase [ADP-forming]; EC 6:3:1:2, Sigma Type III from sheep brain) and an incubation mixture of Brosnan and Hall (17). The L-1<sup>14</sup>C-glutamine was purified by applying the entire incubation mixture (5 ml) to a column containing 29.5 ml Bio-Rad AG1 x 8 (50-100 mesh, formate form) as the top layer and 12.5 ml Bio-Rex 70 (50-100 mesh, H<sup>+</sup> form) as the bottom layer (ion exchange resins obtained from Bio-Rad Laboratories, Richmond, California). L-1<sup>14</sup>C-glutamine was eluted with water, freeze dried, dissolved in isotonic saline, and stored at -20° C until used. Recoveries of <sup>14</sup>C-label in glutamine averaged 85% using this method. Radiochemical purity of the 1-<sup>14</sup>C-glutamine preparation was confirmed by application of the method described in the next section as well as by a modification of the method of Adam and Simpson (1) as described below.

Two samples of the 1-<sup>14</sup>C-glutamine preparation were used and each was taken up in 0.5 ml H<sub>2</sub>O and added to 0.5 ml of 0.1 M acetate pH 5.0. Glutaminase (0.1 international units) was added to tube No. 1 which was then incubated for 1 hour at 37° C. At the end of the incubation, 0.1 ml of 0.5 M sodium phosphate pH 7.0 was added to tube No. 1 to bring the pH to 7. Tube No. 2 was not incubated with glutaminase but was adjusted to pH 7 with 0.1 ml of 0.5 M sodium phosphate and stored on ice. Samples were applied to separate columns (0.7 x 7.0 cm) of Bio-Rad AG1 x 8 (50-100 mesh, formate form) and washed successively with 10 ml distilled water and 10 ml of 1 M formic acid. Each washing was freeze-dried in



scintillation vials, 15 ml Aquasol 2 (New England Nuclear) added, and radioactivity determined. Contamination of the 1-<sup>14</sup>C-glutamine with neutral compounds was estimated by

$$\frac{\text{tube No. 1 water wash}}{\text{tube No. 2 water wash} - \text{tube No. 1 water wash}}$$

while the contamination of the 1-<sup>14</sup>C-glutamine with charged compounds was estimated by

$$\frac{\text{tube No. 2 formic wash}}{\text{tube No. 1 formic wash} - \text{tube No. 2 formic wash}}$$

Using this method, purities of greater than 95% were obtained for all preparations of 1-<sup>14</sup>C-glutamine.

#### D. Specificity of the Glutamate Decarboxylase Preparation

The preparation of glutamate decarboxylase (L-glutamate-1-carboxylase; EC 4:1:1:15, Sigma Type V from E. coli) used was reported to have a decarboxylase activity with L-glutamine, L-arginine, and L-lysine of < 0.1% compared to the activity towards L-glutamate, and a glutaminase activity of 0.001%. Using a Gilson Differential Respirometer, the decarboxylating activity of the preparation was measured with each of the following groups of amino acids at concentrations of 20 mM in 0.5 M acetate buffer, pH 5.0: (i) alanine, glycine, threonine and serine (ii) valine, leucine and isoleucine (iii) cysteine and methionine (iv) proline, asparagine, and aspartic acid (v) lysine, arginine and histidine (vi) glutamine and (vii) saturated solutions of tyrosine and phenylalanine. No



contaminating decarboxylase activity was found since decarboxylation of the above groups of amino acids occurred at less than 0.5% of the rate obtained using 20 mM glutamate.

The effect of the glutamate decarboxylase preparation on amino acids at the normal plasma levels was also investigated. Fresh arterial plasma samples (0.4 ml) were incubated with glutamate decarboxylase (5 international units) in 2 ml of 0.5 M acetate buffer pH 5.0 for 90 minutes at 37° C. At the end of the incubation, 0.6 ml of 12% HClO<sub>4</sub> was added, the mixture was centrifuged and aliquots of the clear supernatant were adjusted to pH 2.2 with LiOH. Control samples were treated in a similar manner but no glutamate decarboxylase was added. Amino acid levels in both control and treated extracts were then measured using an amino acid analyzer as described previously. Results showed that all amino acids were unaffected by the glutamate decarboxylase treatment except glutamate and asparagine. Pure asparagine samples treated in the same manner also showed that the glutamate decarboxylase preparation reacted with asparagine.

Uniformly labelled L-<sup>14</sup>C-asparagine (0.5 mM) was carried through the assay procedure described below and radioactivity trapped in the center wells was counted. Uniformly labelled L-<sup>14</sup>C-glutamate (0.5 mM) was treated in a similar manner for comparison. Results showed that, after 90 minutes incubation, one fifth of the radioactivity from <sup>14</sup>C-glutamate was recovered while radioactivity in the center wells when <sup>14</sup>C-asparagine was used was at background levels. From these experiments we were confident that the glutamate decarboxylase preparation was not contaminated with other decarboxylase activities or with glutaminase.

Glutaminase (L-glutamine aminohydrolase; EC 3:5:1:2, Sigma Grade V from E. coli) was used in the analysis.

#### E. Assay Method

The assay is carried out in 25 ml Erlenmeyer flasks fitted with center wells suspended from rubber stoppers (Kontes Glass Company). Center wells contain 0.2 ml of NCS tissue solubilizer (Amersham Searle) and filter paper wicks (6). The assay method is outlined in Figure 5.

Blood samples (400  $\mu$ l) were taken from the arterial cannulae directly into heparinized micro test tubes and centrifuged at 12,000 x g for 2 minutes in an Eppendorf micro centrifuge (Brinkman Instruments Inc., Toronto, Ontario). A plasma sample (200  $\mu$ l) thus obtained was added to 2.0 ml acetate buffer (0.5 M, pH 5.0), the flasks fitted with center wells, stoppered, and incubated for 30 minutes at 37° C with shaking. Center wells were removed at the end of the incubation and may be counted to determine  $^{14}\text{CO}_2$  derived from plasma bicarbonate. Glutamic decarboxylase (5 international units) was then added to the flasks, fresh center wells and stoppers fitted, and the flasks incubated again for 30 minutes at 37° C with shaking. Center wells were then counted to determine 1- $^{14}\text{C}$ -label in glutamic acid. Glutaminase (0.5 international units) was then added, fresh center wells and stoppers fitted, and, after a further 1 hour incubation, center wells were counted to determine 1- $^{14}\text{C}$ -label in glutamine. For determination of radioactivity, the outside of the center well was carefully wiped with tissue and the entire center well was then added to a scintillation vial containing 10 ml of Toluene/Omnifluor (New

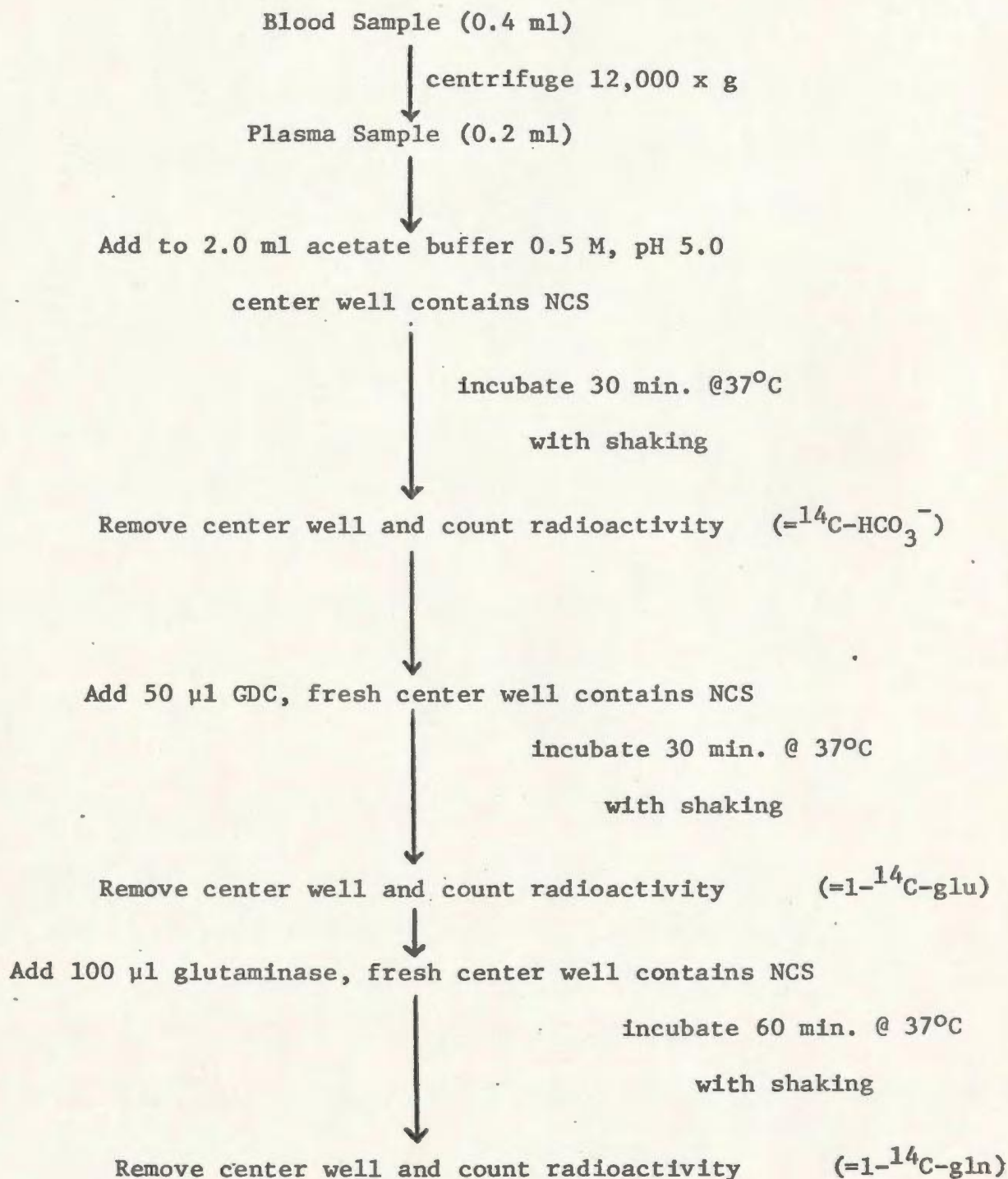
### Legend for Figure 5

$^{14}\text{C-HCO}_3$	=	$^{14}$ carbon label in bicarbonate
$1\text{-}^{14}\text{C glu}$	=	$^{14}$ carbon label in the No. 1 carbon position of glutamate
$1\text{-}^{14}\text{C gln}$	=	$^{14}$ carbon label in the No. 1 carbon position of glutamine
GDC	=	glutamic decarboxylase
NCS	=	NCS tissue solubilizer



FIGURE 5

FLOWCHART FOR THE DETERMINATION OF RADIOACTIVITY IN  
1-<sup>14</sup>C-GLUTAMATE AND 1-<sup>14</sup>C-GLUTAMINE IN PLASMA SAMPLES



England Nuclear). Samples were counted in a refrigerated Beckman LS-233 liquid scintillation counter. Quench correction was made by the channels ratio method; quenching was found to be constant between samples.

Excellent reproducibility and quantitative recovery of added labelled compounds was obtained using this technique.  $U^{14}C$ -glutamine (New England Nuclear), used in the first series of tests, was initially purified by the ion exchange method of Adam and Simpson (1), and added to fresh arterial plasma. Samples were then analyzed for  $1-^{14}C$ -glutamine label by the method just described and values obtained were compared to radioactivity measured by pipetting 0.1 ml samples of plasma into 15 ml Aquasol 2 in scintillation vials. The results (Table 1) show the excellent recovery of label as well as excellent reproducibility of the assay. Excellent reproducibility was also obtained using plasma samples taken from rats that had been infused 1 to 2 hours with  $1-^{14}C$ -glutamine. Results from 10 determinations on a single plasma sample showed  $^{14}C$ -levels of  $2449 \pm 19$  (S.D.) cpm/0.2 ml plasma.

#### F. Whole Body Analysis

Whole body analysis for glutamine and other amino acids was performed on normal and acidotic rats in order to measure the size of the total body glutamine pool. This information was hoped to be of some aid in the interpretation of the data from the turnover studies.

Rats, which had had food withheld for four hours, were weighed, anaesthetized with inactin and taped to a cutting board. The rat was then quickly sectioned into approximately 30 g pieces with a heavy knife

TABLE 1

Recovery of  $^{14}\text{CO}_2$  from  $\text{U}^{14}\text{C}$ -Glutamine Added to a Fresh  
Arterial Plasma Sample

$\text{U}^{14}\text{C}$ -Glutamine Added (cpm/0.1 ml plasma)	$^{14}\text{CO}_2$ Recovered* (cpm/0.1 ml plasma)	% Recovery
143,580 $\pm$ 5300 (6)	149,160 $\pm$ 1141 (10)	103.9 $\pm$ 4.0

Values are repeated as mean  $\pm$  S.D. The number of determinations is indicated in parenthesis.

\* These values were multiplied by 5 since only one-fifth of the label in  $\text{U}^{14}\text{C}$ -glutamine was detected by this assay method.



and the pieces immediately frozen in liquid nitrogen in a tared ice bucket. Frozen pieces were then selected, wrapped in clean, heavy cotton cloth, broken into small fragments with a heavy hammer and returned to the liquid nitrogen. Recovery of tissue, determined by reweighing the ice bucket and crushed tissue together, averaged better than 95%. Crushed tissue was then homogenized in the cold room in approximately 1500 ml of 6%  $\text{HClO}_4$ . A one-gallon waring blender was used, and a good homogenate was obtained by 5 x 1 min spins at top speed. Recovery from this stage was calculated as the difference in the volume of homogenate and the volume of  $\text{HClO}_4$  used. Recoveries from all stages of the extraction process averaged better than 90%.

Extracts were next prepared for complete amino acid analysis. The homogenate was filtered through 4 layers of cheesecloth and centrifuged in an Eppendorf microfuge (12,000 x g for 2 min). Clear extracts were then adjusted to pH 2.2 and amino acid analysis performed as described.

#### IV. PRESENTATION AND ANALYSIS OF DATA

Data were reported as mean  $\pm$  S.D. (standard deviation) with the sample size (n) in parenthesis. Differences between populations were tested for statistical significance by t-test. Arterial and venous samples were obtained from the same rat when possible. A paired t-test was used for these data. P values of 0.05 or less were taken to indicate a significant difference between populations.

## RESULTS

### I. AMINO ACID METABOLISM BY THE KIDNEY

#### A. Whole Blood and Plasma Amino Acid Levels

Amino acid levels in whole blood and plasma obtained from normal and acidotic rats were measured to determine if differences exist. Results are reported in Table 2 and illustrated in Figure 6. In these experiments, whole blood and plasma samples were taken from the same rats and the data obtained are thus paired.

Levels of glycine, glutamic acid, lysine and arginine were lower in plasma than in whole blood in both normal and acidotic rats. These amino acids therefore must be concentrated within the blood cells.\* Further, this concentration gradient is independent of acid-base status of the rat. Changes in both whole blood and plasma amino acid levels occur in acidosis. Levels of serine, threonine and lysine were higher and levels of glutamine were lower in both whole blood and plasma during acidosis. As a result, the total free amino acid level in blood was increased in acidosis.

#### B. Arterio-Venous Differences Across the Kidney for All Amino Acids

Measurements of arterio-venous differences must be made using whole blood to take into account the possible involvement of red blood cells in the transport of metabolites. Arterio-renal venous differences for nineteen amino acids using whole blood are reported in Table 3 and illustrated in Figure 7. These measurements were made so as to quantitate the net metabolism of the various amino acids by the normal and acidotic

\* In subsequent discussion, where "erythrocytes" or "red blood cells" have been mentioned, it should read "blood cells", since our experiments do not distinguish between the different types of blood cells.



TABLE 2

## Amino Acid Levels in Arterial Whole Blood and Plasma from Normal and Acidotic Rats

Results are expressed as  $\mu\text{moles/ml}$  of whole blood or of plasma  $\pm$  S.D.  $n = 4$  in all cases. BLD represents Below Limits of Detection.

	NORMAL			ACIDOTIC			
	Blood Levels	Plasma Levels	P Blood Different from Plasma	Blood Levels	Plasma Levels	P Blood Different from Plasma	P Acidotic Blood Different from Normal Blood
Glycine	$0.28 \pm 0.05$	$0.18 \pm 0.02$	$<0.025$	$0.33 \pm 0.07$	$0.21 \pm 0.01$	$<0.05$	NS
Alanine	$0.36 \pm 0.08$	$0.42 \pm 0.06$	NS	$0.44 \pm 0.06$	$0.47 \pm 0.06$	NS	NS
Serine	$0.21 \pm 0.03$	$0.20 \pm 0.03$	NS	$0.28 \pm 0.03$	$0.27 \pm 0.03$	NS	$<0.001$
Threonine	$0.25 \pm 0.02$	$0.26 \pm 0.02$	NS	$0.39 \pm 0.01$	$0.43 \pm 0.11$	NS	$<0.001$
Leucine	$0.12 \pm 0.01$	$0.13 \pm 0.02$	NS	$0.11 \pm 0.01$	$0.12 \pm 0.01$	NS	NS
Isoleucine	$0.08 \pm 0.01$	$0.09 \pm 0.01$	NS	$0.07 \pm 0.01$	$0.08 \pm 0.01$	NS	NS
Valine	$0.16 \pm 0.01$	$0.18 \pm 0.02$	NS	$0.14 \pm 0.01$	$0.15 \pm 0.01$	NS	NS
Proline	$0.20 \pm 0.03$	$0.19 \pm 0.02$	NS	$0.26 \pm 0.06$	$0.22 \pm 0.02$	NS	NS
Tyrosine	$0.07 \pm 0.02$	$0.08 \pm 0.01$	NS	$0.08 \pm 0.01$	$0.09 \pm 0.01$	NS	NS
Phenylalanine	$0.05 \pm 0.01$	$0.05 \pm 0.01$	NS	$0.05 \pm 0.01$	$0.06 \pm 0.01$	NS	NS
Methionine	BLD	$0.04 \pm 0.01$		BLD	$0.06 \pm 0.00$		
Aspartic Acid	$0.03 \pm 0.01$	BLD		$0.02 \pm 0.01$	BLD		
Glutamic Acid	$0.20 \pm 0.01$	$0.11 \pm 0.02$	$<0.001$	$0.17 \pm 0.02$	$0.10 \pm 0.02$	$<0.010$	NS
Asparagine	$0.06 \pm 0.01$	$0.05 \pm 0.01$	NS	$0.08 \pm 0.01$	$0.06 \pm 0.01$	NS	NS
Glutamine	$0.54 \pm 0.04$	$0.59 \pm 0.09$	NS	$0.38 \pm 0.04$	$0.41 \pm 0.02$	NS	$<0.005$
Lysine	$0.51 \pm 0.05$	$0.39 \pm 0.05$	$<0.05$	$0.77 \pm 0.04$	$0.62 \pm 0.03$	$<0.005$	$<0.001$
Ornithine	$0.06 \pm 0.01$	$0.07 \pm 0.00$	NS	$0.07 \pm 0.01$	$0.05 \pm 0.00$	NS	NS
Histidine	$0.06 \pm 0.01$	$0.07 \pm 0.00$	NS	$0.07 \pm 0.00$	$0.08 \pm 0.00$	NS	NS
Arginine	$0.29 \pm 0.04$	$0.20 \pm 0.04$	$<0.05$	$0.34 \pm 0.04$	$0.21 \pm 0.07$	$<0.050$	NS



# Arterial Blood and Plasma Amino Acid Levels in Normal Rats

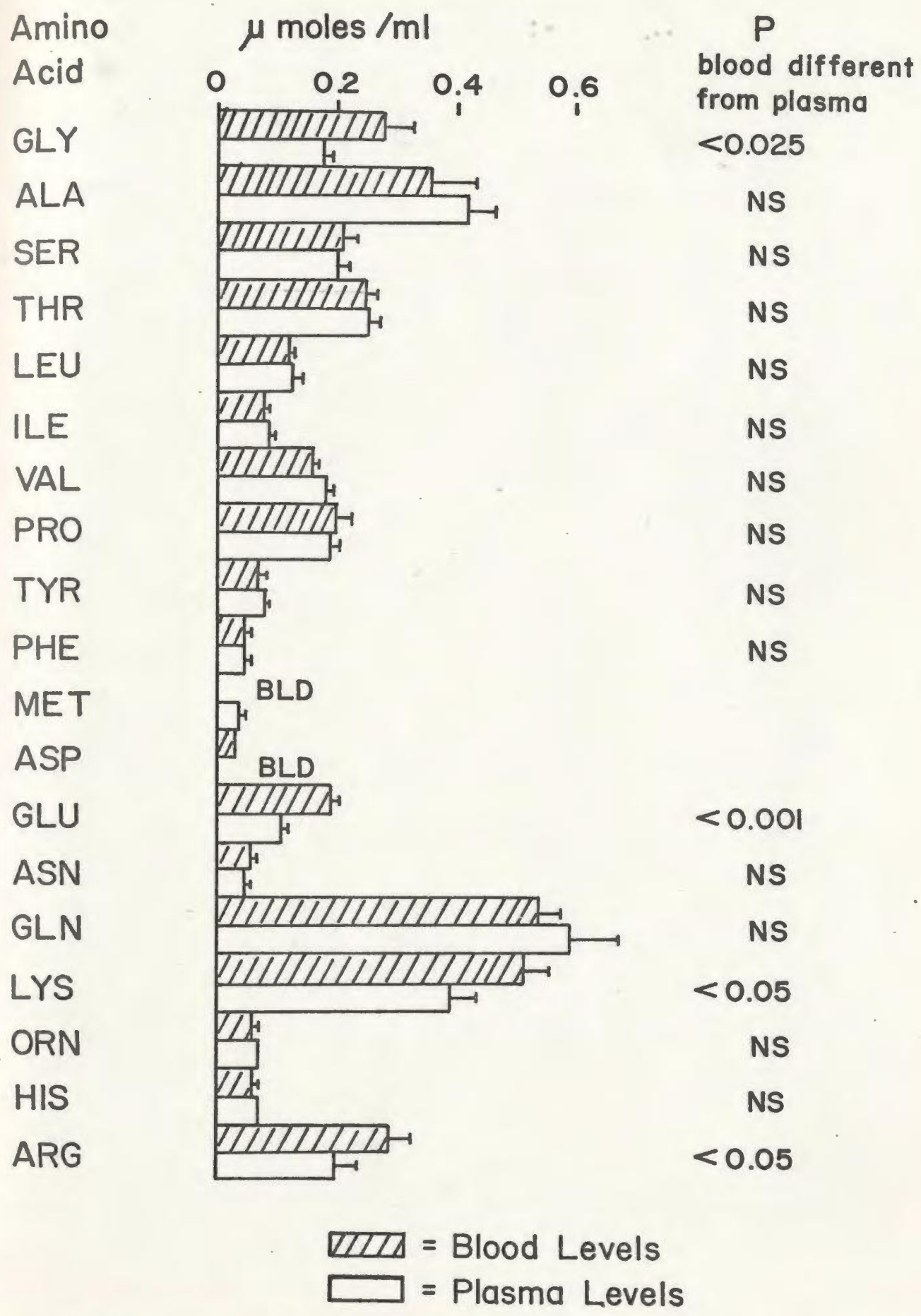


FIG. 6. Arterial Blood and Plasma Amino Acid Levels in Normal Rats.

TABLE 3

Arterial Amino Acid Levels and Arterio-Renal Venous (A-V) Differences in Normal and Acidotic Rats.

Results are expressed as  $\mu\text{moles/ml blood} \pm \text{S.D.}$   $n = 6$ , except where noted. A positive difference implies an uptake by the kidney.

Amino Acid	NORMAL			ACIDOTIC		
	Arterial	A-V	P Value A-V Different from Zero	Arterial	A-V	P Value A-V Different from Zero
Glycine	$0.39 \pm 0.13$	$+0.05 \pm 0.13$	NS	$0.47 \pm 0.08$	$+0.04 \pm 0.06$	NS
Alanine	$0.46 \pm 0.15$	$0.00 \pm 0.08$	NS	$0.46 \pm 0.03$	$+0.01 \pm 0.02$ (4)	NS
Serine	$0.20 \pm 0.05$	$-0.06 \pm 0.03$	<.025	$0.33 \pm 0.07$	$-0.05 \pm 0.02$	<.005
Threonine	$0.25 \pm 0.05$	$-0.03 \pm 0.04$	NS	$0.43 \pm 0.08$	$-0.01 \pm 0.01$ (5)	NS
Leucine	$0.12 \pm 0.02$	$-0.01 \pm 0.02$	NS	$0.11 \pm 0.02$	$0.00 \pm 0.01$	NS
Isoleucine	$0.07 \pm 0.01$	$-0.01 \pm 0.01$	NS	$0.06 \pm 0.02$	$0.00 \pm 0.01$	NS
Valine	$0.17 \pm 0.03$	$-0.02 \pm 0.03$	NS	$0.16 \pm 0.03$	$0.00 \pm 0.01$ (5)	NS
Proline	$0.22 \pm 0.09$	$+0.02 \pm 0.06$	NS	$0.22 \pm 0.04$	$-0.01 \pm 0.02$	NS
Tyrosine	$0.08 \pm 0.02$	$-0.01 \pm 0.01$	NS	$0.10 \pm 0.02$	$-0.01 \pm 0.01$	NS
Phenylalanine	$0.05 \pm 0.01$	$0.00 \pm 0.01$	NS	$0.05 \pm 0.01$	$-0.01 \pm 0.01$	NS
Methionine	$0.30 \pm 0.01$	$-0.01 \pm 0.05$ (5)		$0.02 \pm 0.01$	$-0.01 \pm 0.01$	NS
Aspartic Acid	$0.04 \pm 0.02$	$-0.01 \pm 0.02$		$0.02 \pm 0.02$	$-0.01 \pm 0.02$ (4)	NS
Glutamic Acid	$0.29 \pm 0.07$	$0.00 \pm 0.09$	NS	$0.20 \pm 0.05$	$-0.02 \pm 0.05$ (5)	NS
Asparagine + Glutamine	$0.48 \pm 0.08$	$+0.03 \pm 0.05$	NS	$0.36 \pm 0.05$	$+0.11 \pm 0.05$	<.005
Lysine	$0.45 \pm 0.10$	$+0.00 \pm 0.04$	NS	$0.62 \pm 0.08$	$+0.04 \pm 0.13$	NS
Ornithine	$0.06 \pm 0.02$	$0.00 \pm 0.01$	NS	$0.05 \pm 0.01$	$0.00 \pm 0.01$	NS
Histidine	$0.05 \pm 0.00$	$-0.01 \pm 0.00$	<.005	$0.06 \pm 0.01$	$+0.01 \pm 0.01$	NS
Arginine	$0.23 \pm 0.04$	$-0.02 \pm 0.01$	<.05	$0.32 \pm 0.05$	$+0.01 \pm 0.07$	NS

# Arterio-Venous Differences for Amino Acids Across Kidneys of Normal and Acidotic Rats

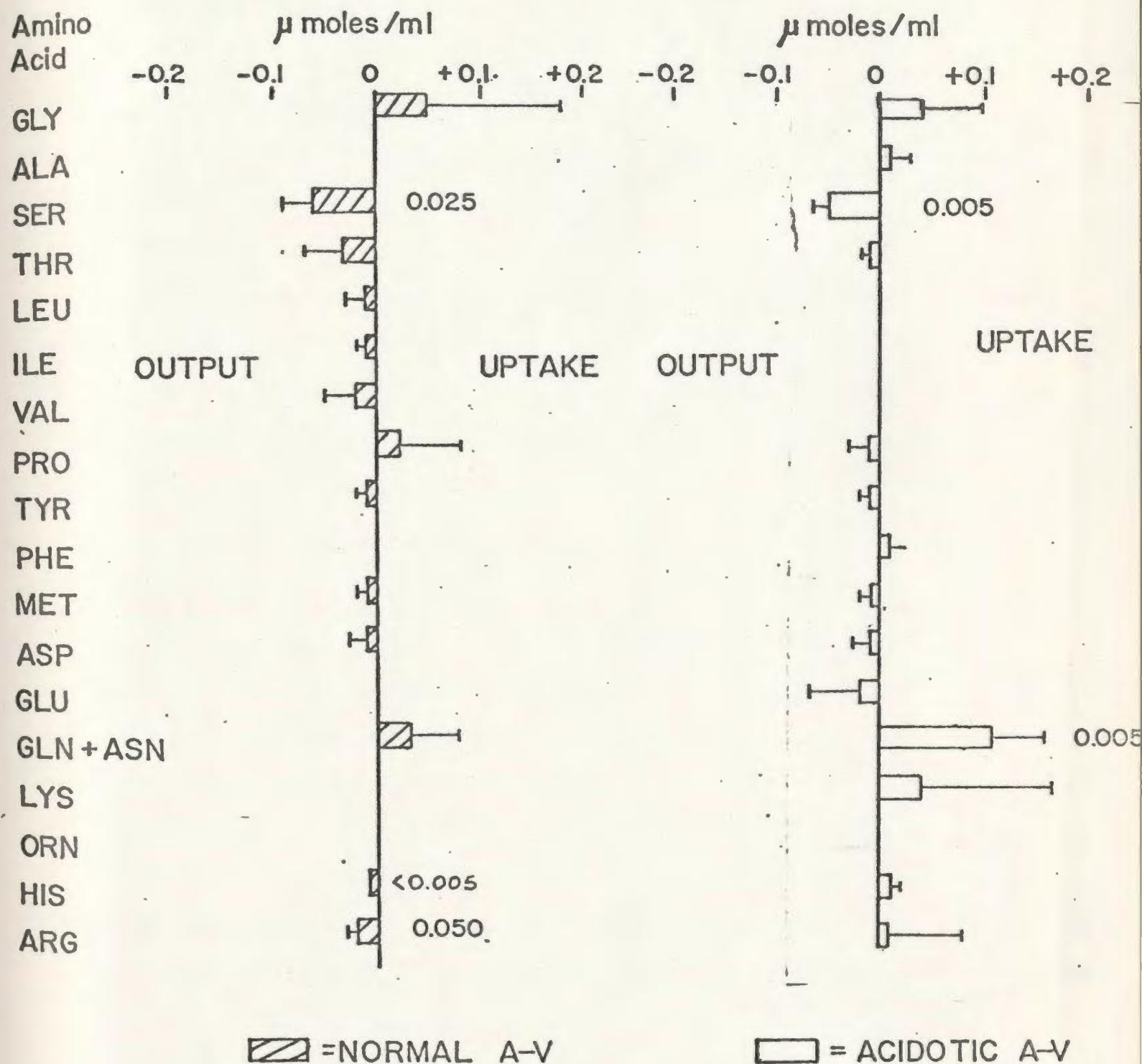


FIG. 7. Arterio-Venous Differences for Amino Acids Across Kidneys of Normal and Acidotic Rats.



rat kidneys. In these experiments, arterial and venous samples were taken from the same rat and, as a consequence, paired statistics were applied to the data.

The results show significant outputs of serine by both the normal and acidotic kidney, as well as small but significant outputs of histidine and arginine by the normal kidney. No amino acids were taken up in significant amounts by the normal kidney. However, the glutamine + asparagine fraction was decreased after passage through the kidney during acidosis. However, experiments reported in Table 4 show that asparagine, which is present at low concentrations in blood, is not removed by the acidotic kidney. Thus, it is glutamine alone that is removed by the acidotic kidney to such a large extent. Measurements of urinary amino acid levels (72) showed that the small amount of glutamine appearing in the urine could in no way account for the glutamine removed from the blood by the kidney.

#### C. Whole Blood and Plasma Kidney Arterio-Venous Differences

Since glutamine was removed from the arterial blood by the acidotic kidney, arterio-renal venous differences were measured using both whole blood and plasma to determine the extent of glutamine and glutamate uptake from the blood cells and plasma (Table 4). Plasma glutamine arterio-venous differences were greater than whole blood glutamine arterio-venous differences ( $P < .005$ ). Haematocrits were measured for normal and acidotic arterial blood and found to be  $43.6 \pm 2.4$  (4) and  $43.7 \pm 3.9$  (6) respectively. Thus since plasma occupies 56.3% of the blood volume of acidotic rats, we may cal-

TABLE 4

Plasma and Whole Blood Levels and Arterio-Venous Differences for  
Glutamine, Glutamate and Asparagine Across Kidneys of Acidotic  
Rats

Results are expressed as  $\mu\text{moles/ml} \pm \text{S.D.}$   $n = 5$  in all cases.

		<u>Blood</u>	<u>Plasma</u>
Glutamine	Renal Vein	$0.37 \pm 0.07$	$0.33 \pm 0.11$
	Artery	$0.46 \pm 0.03$	$0.50 \pm 0.08$
	A-V	$0.10 \pm 0.05$	$0.17 \pm 0.07$
	P for A-V Different from Zero	$<0.025$	$<0.005$
Glutamate	Renal Vein	$0.15 \pm 0.01$	$0.06 \pm 0.02$
	Artery	$0.14 \pm 0.02$	$0.08 \pm 0.02$
	A-V		$0.03 \pm 0.01$
	P for A-V Different from Zero	NS	$<0.005$
Asparagine	Renal Vein	$0.05 \pm 0.01$	$0.05 \pm 0.01$
	Artery	$0.05 \pm 0.01$	$0.05 \pm 0.01$
	A-V		
	P for A-V Different from Zero	NS	NS

culate that for every millilitre of blood that perfuses these kidneys in vivo 0.096  $\mu$ mole of glutamine ( $0.533 \times 0.170$ ) (kidney arterio-venous difference for plasma glutamine is 0.170  $\mu$ mole/ml, Table 4) is, on the average, extracted from the plasma. Similarly, for every millilitre of blood that perfuses these kidneys in vivo a total of 0.095  $\mu$ mole of glutamine ( $1 \times 0.095$ ) (kidney arterio-venous difference for blood glutamine is 0.095  $\mu$ mole/ml, Table 4) is extracted from the blood. This remarkable agreement in values indicates that glutamine taken up by the acidotic kidney is removed predominantly from the plasma and that the erythrocyte glutamine levels remain unchanged as the blood passes through the kidney. In contrast, while the whole blood arterio-venous difference for glutamate showed no uptake of glutamate by the kidney, arterial plasma levels were significantly elevated above the renal venous plasma levels. Thus, glutamate moves from the plasma into the erythrocytes as the blood perfuses the kidney. There was no significant change in the concentration of asparagine in whole blood or plasma after passage through the kidney.

#### D. Metabolism of Lactate by the Kidney

In a further study of kidney metabolism, arterio-venous differences for lactate were measured across the normal and acidotic rat kidney. This study was undertaken to determine whether lactate, which is an important fuel of the normal kidney, is replaced by glutamine, in the acidotic kidney. Since in the dog total  $\text{CO}_2$  production does not differ in acidosis (46) one might expect that if glutamine carbon is oxidized to  $\text{CO}_2$  in the acidotic rat kidney, the uptake and oxidation of



lactate for energy production may be reduced. Results reported in Table 5, however, show that kidneys from both normal and acidotic rats removed comparable quantities of lactate from the blood. In addition, there was no significant difference in arterial lactate concentrations between normal and acidotic rats.

## II. GLUTAMINE METABOLISM IN THE WHOLE RAT

The remainder of the experiments performed deal with measurements of the overall metabolism of glutamine in the entire rat. Experiments were performed in the hope of detecting the changes which occur in overall glutamine metabolism in acidosis.

### A. Measurement of the Turnover Rate of Glutamine

The first series of experiments involved the measurement in vivo of the turnover rate of plasma glutamine in normal and acidotic rats. Since Table 3 unequivocally demonstrated increased renal glutamine extraction in the rat it was felt that turnover studies would give information on the "source" of this glutamine. Thus, if extra glutamine were synthesized during acidosis one should see an increased glutamine turnover. However, if the renal glutamine were provided via a decreased glutamine utilization by other tissues, then there should be no change in glutamine turnover. As outlined in the Introduction, two different techniques were used in the measurement of turnover rates, namely single injection and constant infusion.

TABLE 5

Arterial Blood Lactate Concentrations and Renal Arterio-Venous Differences  
of Lactate in Normal and Acidotic RatsResults are expressed as  $\mu\text{moles/ml} \pm \text{S.D.}$ 

	Normal (6)	Acidotic (9)	P (normal different from acidotic)
Arterial	$1.65 \pm 0.46$	$1.63 \pm 0.38$	NS
A-V Difference	$+0.31 \pm 0.08$	$+0.32 \pm 0.22$	NS
P for A-V Different from Zero	$<0.001$	$<0.005$	

A typical set of results obtained from a single injection experiment is plotted in Figure 3 (page 33). After an initial rapidly changing rate of decline in  $\log_{10}$  of plasma glutamine specific activity, a linear rate of decrease with time was obtained. As previously discussed in the Introduction, the slope of the linear portion of the plot as well as the y-intercept are used in the calculation of turnover rate and pool size.

A typical set of results obtained by using the constant infusion technique is plotted in Figure 8. Note that a plateau was quickly established in plasma glutamine specific activity, indicating that the label has equilibrated throughout the pool to a constant specific activity. From the position of this plateau as well as the rate of infusion of label, the turnover rate can be calculated.

The turnover rates of  $1\text{-}^{14}\text{C}$  glutamine in normal and acidotic rats measured by both techniques are reported in Table 6. There was no difference in turnover rates of glutamine between normal and acidotic rats as measured by both methods. However, rates measured by single injection were more than twice those measured by the constant infusion technique.

Values for the pool size of glutamine calculated from the single injection experiments also appear in Table 6. Similar values were obtained for normal and acidotic rats. Experiments were now performed to determine how closely the calculated pool size approximates the total body glutamine pool. In addition, differences in whole body levels of various amino acids were measured between normal and acidotic rats.



### Legend for Figure 8

Shown here is a typical plot of the data obtained by the constant infusion technique. In this particular experiment an acidotic rat was used (wt = 332 g).  $1\text{-}^{14}\text{C}$  glutamine ( $8.16 \times 10^6$  cpm/ml) was infused at a rate of  $1.54 \times 10^7$  cpm/hr.

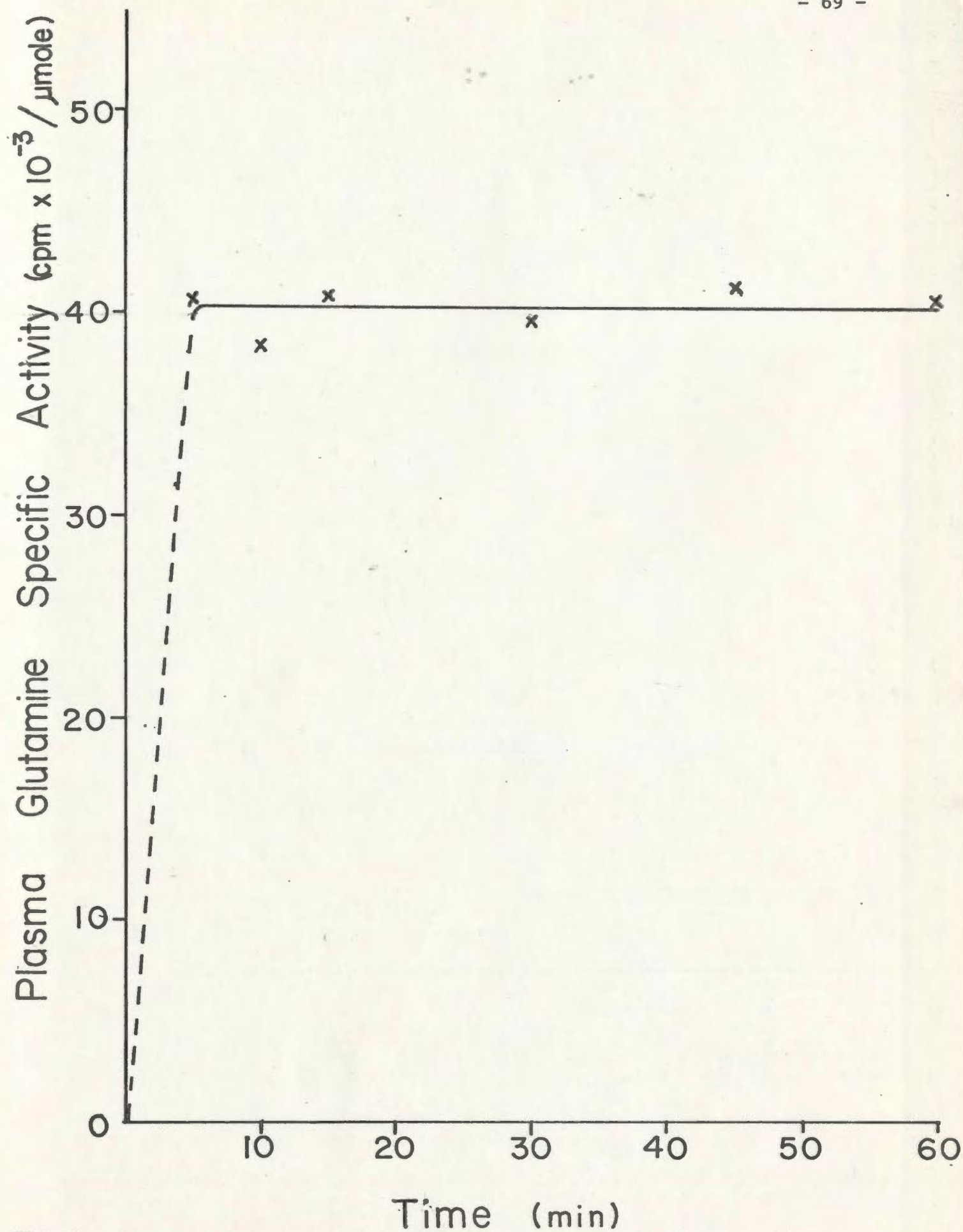


FIG. 8. Constant Infusion Method for the Measurement of Glutamine Turnover.

TABLE 6

Turnover Rates of 1-<sup>14</sup>C-Glutamine in Normal and Acidotic Rats

	Single Injection	Constant Infusion	Pool Size (from single injection studies (umoles/100 g)
Normal	282.4 ± 32.6 (4)	133.8 ± 8.4 (5)	130.6 ± 31.6
Acidotic	308.8 ± 35.5 (5)	131.2 ± 27.4 (5)	120.2 ± 15.5
P (normal different from acidotic)	NS	NS	NS

Values for turnover rates are in umoles/hr/100 g body wt. Numbers in parenthesis refer to the number of experiments performed in each group.



## B. Measurement of Whole Body Amino Acid Levels

Whole body extracts were prepared as described in the methods section and the concentrations of amino acids are reported in Table 7 and illustrated in Figure 9. Whole body glutamine levels are approximately twice as high as the pool size calculated from the single injection studies. It thus seems that plasma glutamine does not freely equilibrate with all tissues. The turnover rates reported are thus for a glutamine pool which comprises only one-half of the total body glutamine. The actual tissues which comprise this pool are not known.

Differences were detected in whole body levels of some amino acids between normal and acidotic rats. Whole body levels of two hydroxy-amino acids, serine and threonine as well as three basic amino acids, lysine, histidine and arginine, as well as citrulline were higher in acidotic than normal rats. However, only the increased levels of serine, threonine, and lysine were reflected in increased blood levels of these amino acids during acidosis.

Other workers have measured glutamine levels in various tissues which have been rapidly freeze-clamped after removal from the animal. Glutamine levels measured by this technique may therefore more closely approximate actual levels in vivo. It is of interest therefore to estimate the whole body pool of glutamine by a summation of these data. Despite the considerable assumptions in the calculation it is remarkable that the calculated whole body glutamine (Table 8) agrees so well with the experimentally determined value (Table 7).

TABLE 7

Concentrations of Amino Acids and Other Ninhydrin Positive Substances in Whole Body Extracts of Normal and Acidotic Rats

Results are expressed as  $\mu\text{moles}/100 \text{ g body wt} \pm \text{S.D.}$   $n = 4$  except where indicated.

	Normal	Acidotic	P (Acidotic different from Normal)
Glycine	305.7 $\pm$ 36.6	318.3 $\pm$ 24.3	NS
Alanine	146.9 $\pm$ 2.7	146.8 $\pm$ 7.7	NS
Serine	105.2 $\pm$ 7.8	142.6 $\pm$ 8.0	<.005
Threonine	65.3 $\pm$ 6.4	95.9 $\pm$ 5.7	<.001
Leucine	15.1 $\pm$ 2.7	13.5 $\pm$ 2.1	NS
Isoleucine	11.1 $\pm$ 2.7	10.3 $\pm$ 1.8	NS
Valine	15.2 $\pm$ 5.1	16.3 $\pm$ 3.7	NS
Proline	26.0 $\pm$ 3.2	31.0 $\pm$ 2.0	NS
Tyrosine	12.8 $\pm$ 2.9	15.3 $\pm$ 1.8	NS
Phenylalanine	7.1 $\pm$ 1.2	7.2 $\pm$ 0.5	NS
Methionine	6.5 $\pm$ 1.5	7.8 $\pm$ 0.6	NS
Glutamic Acid	188.0 $\pm$ 10.9	174.8 $\pm$ 9.3	NS
Asparagine	13.1 $\pm$ 2.0	13.7 $\pm$ 1.4	NS
Glutamine	263.8 $\pm$ 11.5	243.9 $\pm$ 26.6	NS
Lysine	57.4 $\pm$ 13.2	117.9 $\pm$ 12.6	<.005
Ornithine	6.7 $\pm$ 2.0	7.0 $\pm$ 1.4	NS
Histidine	23.0 $\pm$ 3.7	31.5 $\pm$ 1.5	<.025
Arginine	56.5 $\pm$ 5.6	95.9 $\pm$ 10.6	<.005
Hydroxyproline	11.4 $\pm$ 5.7	8.5 $\pm$ 1.4 (3)	NS
Taurine	997.7 $\pm$ 78.0 (3)	958.5 $\pm$ 25.9	NS
Citrulline	10.6 $\pm$ 1.2	14.3 $\pm$ 1.3	<.025
Carnosine	164.0 $\pm$ 39.6	151.0 $\pm$ 8.6	NS
Anserine	257.6 $\pm$ 63.2	246.4 $\pm$ 17.7	NS

# Whole Body Amino Acid Levels in the Rat

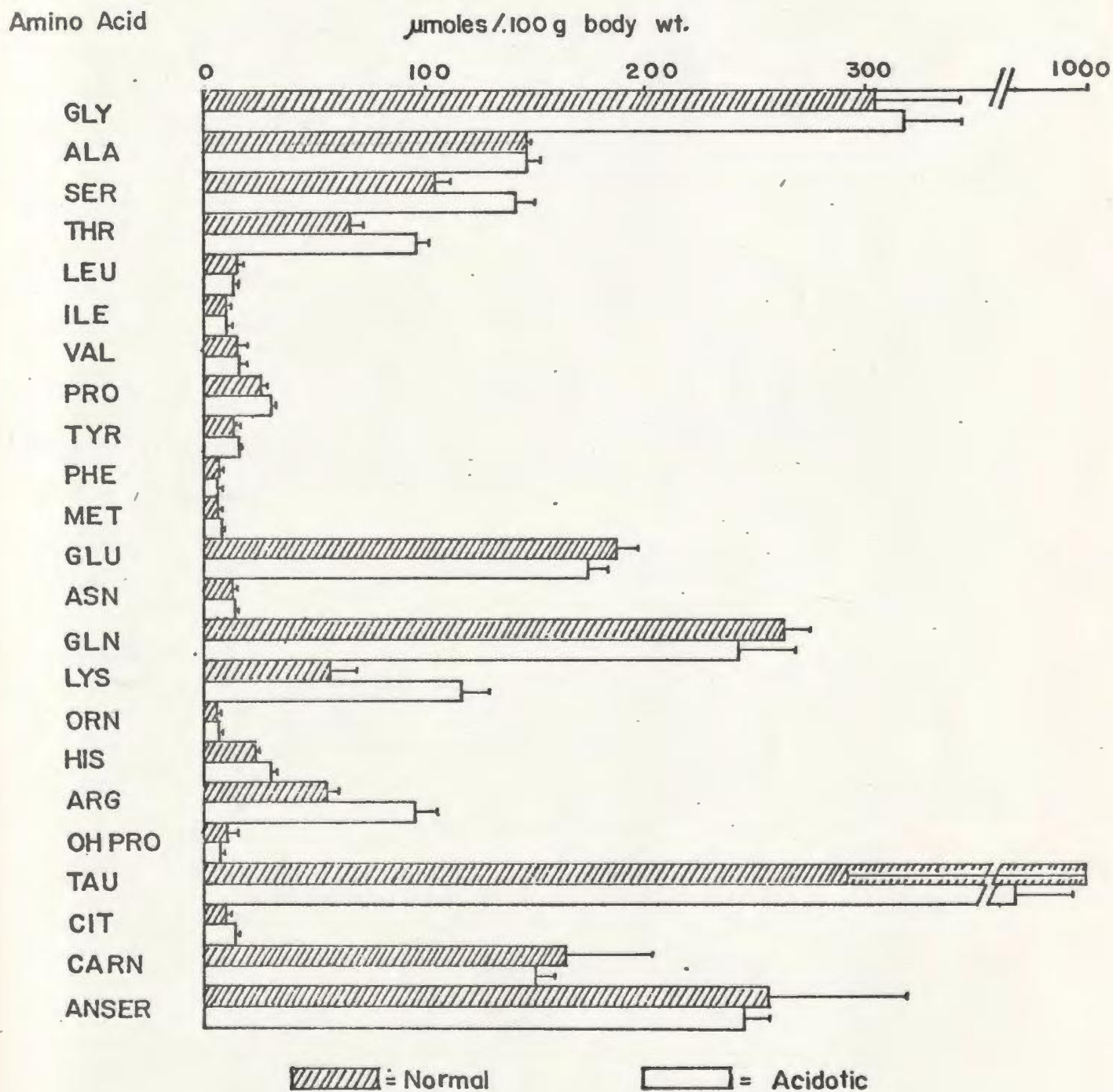


FIG. 9. Whole Body Amino Acid Levels in the Rat.



TABLE 8

## Glutamine Levels in Various Tissues of the Rat

Tissue	Ave Gln Content ( $\mu$ moles/g tissue)	Ref	Percentage of Body wt (g%) (1)	Ave Gln Content ( $\mu$ moles/100 g body wt)
Muscle	3.3	3	41.7	137.6
Skin	2.0	*	18.0	36.0
Blood	0.57	5	8.0	4.6
Liver	2.68	4	2.3	6.2
Brain	4.51	5	2.0	9.0
Intestine	0.55	3	1.8	1.0
Lungs	1.12	4	0.7	0.8
Heart	5.99	4	0.5	3.0
Kidney	1.72	2	0.4	0.7
Spleen	1.27	4	0.2	0.3
Adipose Tissue	-	*	0-10	0
Skeleton	-	*	15.9	0

Total Gln Content = 199.1

\* Estimated values.

1. Pitts, R.F. "Physiology of the Kidney and Body Fluids", 3rd ed, pl1, Year Book Medical Publishers, Chicago, (1974). (Values for a 70 kg man).
2. Brosnan, J.T. D.Phil thesis. Oxford University (1968).
3. Windmueller, H.G. and Spaeth, A.E. J. Biol. Chem. 249; 5070-5079 (1974).
4. Herbert, J.D., Coulson, R.A. and Hernandez, T. Comp. Biochem. Physiol. 17, 583-598 (1966).
5. Lund, P. In "Methods of Enzymatic Analysis", 2nd ed, vol. 4, Ed. H.U. Bergmeyer, p1719-1722, Academic Press (1974).

### C. Arterio-Venous Differences for Glutamine and Glutamate Across all Organs

The final set of experiments involve the direct measurement of arterio-venous differences across various organs in the intact rat. These were performed in an attempt to obtain direct information on the sites of glutamine utilization and provision in the rat. Since, in these experiments blood samples from the various veins and the arteries were obtained from the same rats, the data obtained were paired and paired statistics were therefore applied.

The data for glutamine arterio-venous differences are reported in Table 9 and illustrated in Figure 10. In the normal rat, glutamine is produced by the muscle (A-FV difference). No significant uptake of glutamine by any tissue was observed. Experiments performed by other investigators have, however, showed that the intestine normally removes glutamine from the blood (82). In addition, previous work (72) yielded unpaired data which showed an uptake of glutamine by the G.I.T. of the normal rat. Apparently the larger random errors in these data have masked any significant arterio-venous difference across the G.I.T. which may be obtained. In acidosis, the kidney removes approximately 40% of the total arterial glutamine in each pass through the kidney. The intestinal bed also removes glutamine, but the A-PV difference is much less than the A-RV difference. The site of glutamine production in the acidotic rat is not apparent from these data. The muscle may produce glutamine in acidosis as well as in normal acid-base status. However, there did not seem to be increased glutamine production by muscle during acidosis, although the variability in the data was rather large.

TABLE 9

Whole Blood Glutamine Levels and Arterio-Venous Differences Across  
Various Tissues of the Rat

	<u>Normal</u>			<u>Acidotic</u>		
	Blood Levels	A-V	P (Vein Different from Artery)	Blood Levels	A-V	P (Vein Different from Artery)
Jugular Vein	0.420 $\pm$ .050	-0.026 $\pm$ .045	NS	0.323 $\pm$ .086	-0.009 $\pm$ .056	NS
Femoral Vein	0.455 $\pm$ .059	-0.061 $\pm$ .016	<.001	0.342 $\pm$ .115	-0.028 $\pm$ .099	NS
Portal Vein	0.357 $\pm$ .050	0.038 $\pm$ .042	NS	0.287 $\pm$ .065	0.027 $\pm$ .020	<.025
Renal Vein	0.397 $\pm$ .048	-0.003 $\pm$ .053	NS	0.184 $\pm$ .064	0.130 $\pm$ .054	<.001
Hepatic Vein	0.387 $\pm$ .069	0.008 $\pm$ .049	NS	0.287 $\pm$ .044	0.027 $\pm$ .046	NS
Arterial	0.395 $\pm$ .054			0.314 $\pm$ .070		

n = 6 in all cases, values are expressed in  $\mu$ moles/ml blood.



# Whole Blood Glutamine Levels in Blood Vessels of the Rat

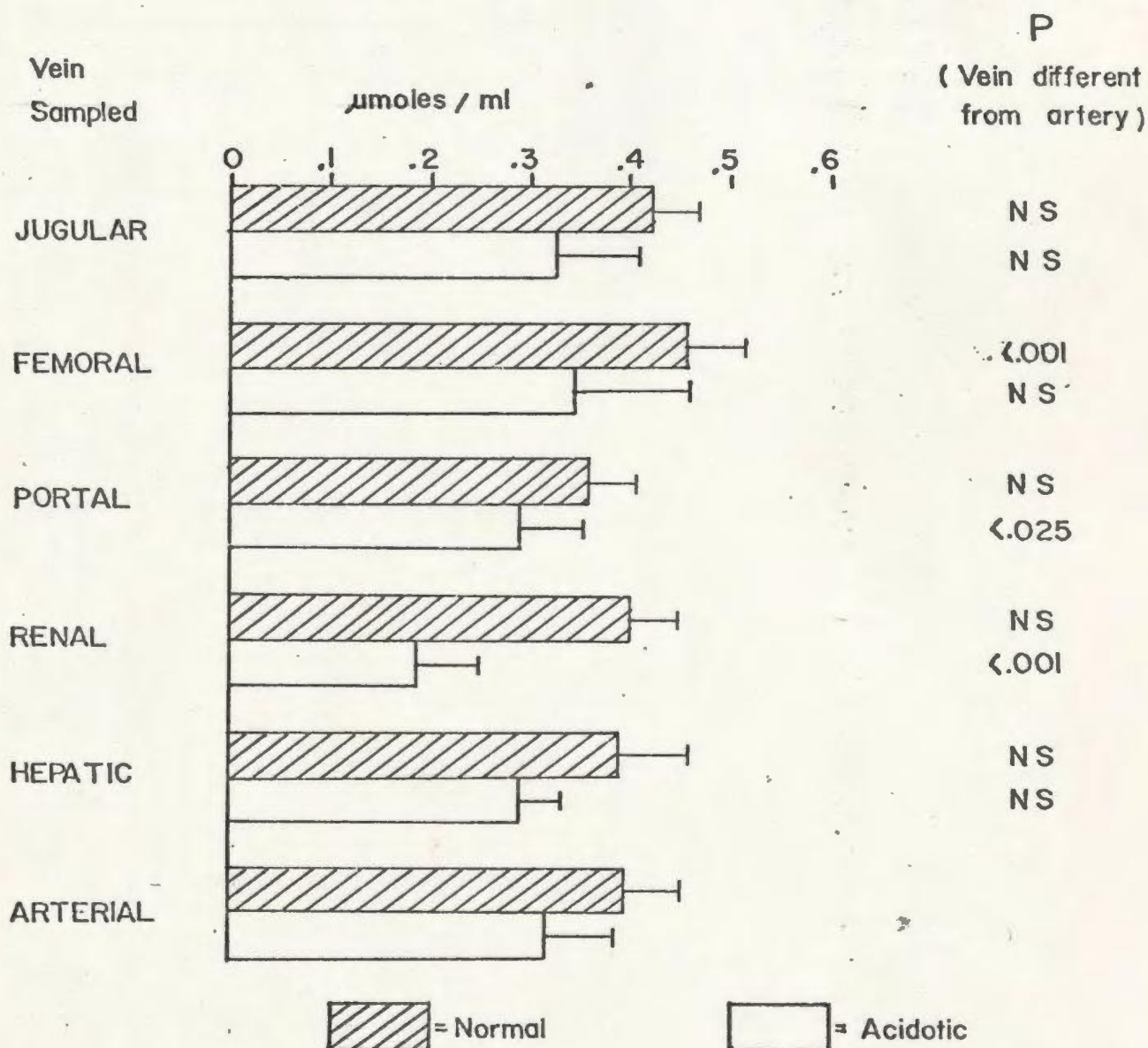


FIG. 10. Whole Blood Glutamine Levels in Blood Vessels of the Rat.

Arterio-venous differences for glutamate were also measured on these same rats. Since glutamate is a precursor of glutamine, it was of interest to determine if glutamine production and glutamate uptake were closely related. Results are reported in Table 10 and illustrated in Figure 11, and show, however, no uptake of glutamate by glutamine producing tissues. In fact, the muscle and brain of the normal rat release glutamate. The G.I.T. also releases glutamate; however this release was only found to be significant during acidosis. Glutamate released by the G.I.T. may be derived from the metabolism of glutamine.

TABLE 10

Whole Blood Glutamate Levels and Arterio-Venous Differences Across  
Various Tissues of the Rat

	<u>Normal</u>			<u>Acidotic</u>		
	<u>Blood Level</u>	<u>A-V</u>	<u>P</u>	<u>Blood Level</u>	<u>A-V</u>	<u>P</u>
Jugular Vein	0.176 $\pm$ .010	-0.024 $\pm$ .019	<.05	0.128 $\pm$ .026	-0.002 $\pm$ .031	NS
Femoral Vein	0.163 $\pm$ .013	-0.011 $\pm$ .011	<.05	0.120 $\pm$ .040	0.007 $\pm$ .049	NS
Portal Vein	0.170 $\pm$ .018	-0.018 $\pm$ .022	NS	0.145 $\pm$ .021	-0.018 $\pm$ .016	<.05
Renal Vein	0.147 $\pm$ .017	0.005 $\pm$ .024	NS	0.132 $\pm$ .051	-0.005 $\pm$ .023	NS
Hepatic Vein	0.169 $\pm$ .032	-0.017 $\pm$ .035	NS	0.138 $\pm$ .022	-0.012 $\pm$ .021	NS
Arterial	0.152 $\pm$ .018			0.126 $\pm$ .034		

n = 6 in all cases, values are expressed in umoles/ml blood.



# Whole Blood Glutamate Levels in Blood Vessels of the Rat

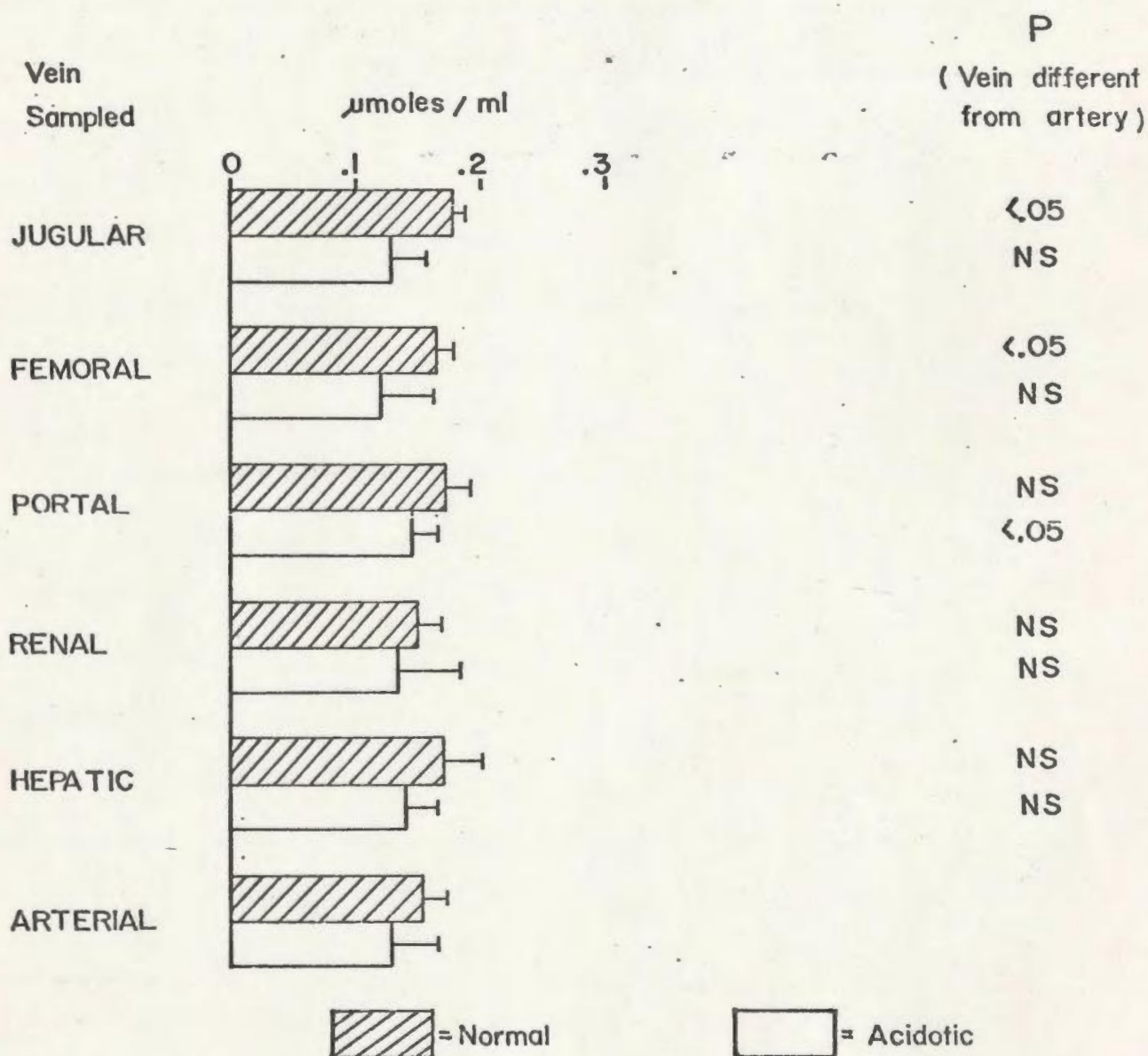


FIG. 11. Whole Blood Glutamate Levels in Blood Vessels of the Rat.

## DISCUSSION

### I. AMINO ACID METABOLISM BY THE KIDNEY

#### A. Whole Blood and Plasma Amino Acid Levels

The measurements of amino acid concentrations in plasma and in whole blood demonstrated consistently lower levels of glycine, glutamate, lysine and arginine in plasma than in whole blood. Such uneven distributions of amino acids across the erythrocyte membrane have previously been reported in blood from dogs (26, 28) and man (7, 8, 30). Although the mechanisms by which these uneven distributions are maintained are uncertain, it is clear that passive distribution in response to Gibbs-Donnan effects is not solely responsible. This inference is based on the observation that a neutral amino acid (glycine), an acidic amino acid (glutamic acid) and two basic amino acids (lysine and arginine) are all concentrated inside the erythrocytes.

Differences were also found in the levels of some amino acids in whole blood and plasma in acidosis. The increased levels of serine, threonine, and lysine observed in both whole blood and plasma of the acidotic rat are reflected in increased whole body levels of these amino acids. However, although no change in the total free glutamine content of rat was observed during acidosis, significantly lower whole blood and plasma glutamine levels were observed in the acidotic rat. Although this decrease has not been observed previously by some investigators (51, 81), others have found significantly decreased levels of glutamine in acidotic sheep (35) and man (56). It may be that the decrease in circulating glutamine levels is due to the increased renal

utilization of this amino acid during acidosis. This has been borne out by a recent observation (58) that as renal extraction of glutamine increases during the onset of acidosis the blood glutamine level falls and as renal extraction of glutamine declines during recovery from acidosis the blood glutamine level returns to normal. The lower levels of glutamine during acidosis may signal a lessening of glutamine utilization by extrarenal tissues.

#### B. Arterio-Venous Differences Across the Kidney

Arterio-venous differences for all amino acids across the kidney show significant outputs of serine by both the normal and acidotic kidney as well as small but significant outputs of histidine and arginine by the normal kidney. Renal output of serine has been observed in acidotic and alkalotic dogs (71), in normal and acidotic man (56) and in normal and starved rats (4, 86). That such renal serine production is seen in acidosis and in alkalosis as well as in fed and starved animals indicates that its production is related neither to the acid-base status of the animal nor to the provision of gluconeogenic amino acids. The precursor of this serine is thought to be glycine (60), although in our studies the arterio-venous difference for glycine uptake across the kidney was not statistically significant.

There was no production of alanine by either the normal or acidotic kidney. Renal alanine output has been found in dogs (61, 71), rats (4, 86) and man (56). However, the output in the rat was quite modest, and some workers (18) have reported a small uptake of alanine by the human



kidney. Thus it appears that alanine release is not an inevitable result of renal metabolism.

The only amino acid extracted by the kidney was glutamine, which was removed in significant quantities only during acidosis. It has been reported that rat kidney slices (22) and the perfused rat kidney (59) produce ammonia from amino acids other than glutamine. Such ammonia production has also been shown to be more rapid in slices and in perfused kidneys from acidotic rats. However, from our measurements in vivo it is apparent that glutamine is the major source of urinary ammonia in the acidotic rat. It is probable that both nitrogens of glutamine are employed for ammonia production, since no increase in amino acid release by the kidney occurs during acidosis.

#### C. Permeability of Erythrocytes to Glutamine and Glutamate

The measurement of glutamine arterio-renal venous differences using both whole blood and plasma have indicated that glutamine is removed by the kidney only from the plasma. The very slow movement of glutamine across the erythrocyte membrane is undoubtedly the cause of the non-involvement of the erythrocyte glutamine pool in ammonia production. Measurements in vitro showed that labelled glutamine penetrated the cell membrane extremely slowly and, in addition, the erythrocyte glutamine pool was extremely resistant to depletion by washing (data not shown). Preliminary measurements of the rate of equilibration of labelled glutamine between blood cells and plasma in vivo were inconclusive and proved technically unfeasible due to the fast removal of the label from the blood. However, a slow equilibration between erythrocyte and plasma

glutamine must occur in vivo since the large decrease in blood glutamine during acidosis is equally manifest in plasma and in whole blood. Pitts et al. (61, 63) have also demonstrated in the dog that the kidney removes glutamine primarily from plasma.

Measurements in vitro have also shown that labelled glutamate fails to quickly penetrate the erythrocyte membrane and that intracellular glutamate is very resistant to depletion by washing (data not shown). However, in contrast to the in vivo data obtained with glutamine, there appears to be a rapid movement of glutamate between the erythrocytes and plasma in vivo. Thus, although there was no significant uptake or output of glutamate by the acidotic kidney, the concentration of glutamate in the arterial plasma was significantly higher than in the renal venous plasma. Hence, a movement of glutamate from plasma to erythrocytes occurred as the blood passed through the kidney. A movement of glutamate into erythrocytes has previously been reported by Aoki et al. (8) and Elwyn (26, 28). It is possible that during passage of the blood cells through narrow capillaries, the transport of amino acids may be facilitated in some manner through contact with the capillary walls. It has been suggested (8) that insulin may affect the uptake and release of glutamate by the red blood cells.

#### D. Renal Lactate Metabolism

The induction of acidosis had no effect on either the arterial lactate levels or the renal extraction of lactate. Other authors (88) have reported that, in the acutely acidotic rat, no change in renal



lactate oxidation has been observed in vitro. In contrast, Leal-Pinto et al. (46) reported decreased lactate uptake by the acidotic dog kidney compared to the alkalotic dog kidney. In addition, no difference in  $\text{CO}_2$  production by the kidney was measured in these two acid-base states. However, since no measurement of lactate extraction by the normal dog kidney was made by these authors, it is not possible to say whether more or less lactate is used by the normal dog kidney. Thus, it appears likely that in the acidotic rat kidney glutamine does not replace lactate as a major fuel of respiration. However, the acidotic dog kidney appears to utilize less lactate, presumably due to increased oxidation of glutamine carbon to  $\text{CO}_2$ .

## II. MEASUREMENTS OF THE OVERALL METABOLISM OF GLUTAMINE

### A. Measurement of Turnover Rates

The measurement of the turnover rate of  $1\text{-}^{14}\text{C}$ -glutamine in the rat has involved considerable effort.  $^{14}\text{C}$ -glutamine specifically labelled in the 1-carbon position is not available commercially and I therefore synthesized and purified this compound. Glutamine synthetase (Sigma Type III from sheep brain) was used with the incubation mixture of Brosnan and Hall (17) and the  $1\text{-}^{14}\text{C}$ -glutamine synthesized was made virtually salt-free and pure by the use of ion exchange resins as described in the methods section. It was also necessary to develop a good method for the measurement of the specific activity of the  $1\text{-}^{14}\text{C}$ -glutamine in the blood, since methods available in the literature were not entirely



satisfactory. A completely new and precise method was thus developed using the sequential action of glutamate decarboxylase and glutaminase. Methods were also developed to confirm the purity of the commercial glutamate decarboxylase preparation.

The measurement of the turnover rates of glutamine in the normal and acidotic rat should give valuable insight into the changes which occur in glutamine metabolism during acidosis. The increased renal extraction of glutamine during acidosis (Table 3) demands increased amounts of glutamine to be available to the kidney. This may be accomplished by either increased glutamine production by whatever tissues produce glutamine or decreased glutamine utilization by extrarenal tissues such as the G.I.T. If more glutamine was synthesized during acidosis, an increased rate of overall turnover of glutamine would be expected. However, if a compensatory decrease in utilization of glutamine by extrarenal tissues were to occur in response to increased renal extraction of glutamine, no change in the turnover rate of glutamine would be expected. Since no change in the turnover rate of glutamine was noted by either method, it appears that glutamine production does not increase in acidosis.

(1) Differences between Single Injection and Constant Infusion Values.

Of interest is the large discrepancy in the value of the turnover rate of glutamine as measured by the two different methods. The turnover rate as measured by the single injection technique was always substantially greater than that measured using the constant infusion method. A similar

but substantially smaller discrepancy in the values obtained for the turnover rate of glucose has been reported (37). These authors have reviewed the extensive work done to resolve this problem. The discrepancy comes, according to these authors, from the simple interpretation applied to the single injection data. Thus, the Zilversmit formula discussed earlier in the Introduction utilizes only the monoexponential portion of the  $\ln$  glutamine specific activity versus time curve and assumes a simple two-compartment model.

One can rewrite the Zilversmit formula given previously:

$$\begin{aligned} \text{where } b^* &= \text{mass of injected } 1\text{-}^{14}\text{C-glutamine} \\ B &= \text{mass of glutamine in the pool (i.e., pool size)} \\ V_1 &= \text{turnover rate} \end{aligned}$$

$$\frac{db^*}{b} = \frac{-V_1}{B} dt$$

in the form given by Hetenyi and Norwich (37):

$$\frac{db^*}{dt} = -V_1 \frac{b^*}{B}$$

Solving the equation they get:

$$\ln \frac{b^*}{B} = \frac{-V_1}{B} t + \ln \frac{b_0^*}{B}$$

where  $b_0$  is the specific activity of glutamine at  $t = 0$ .

This equation may be expressed in exponential form:

$$b^*t = b_o^* \exp\left(\frac{-V_1}{B} t\right)$$

Upon integration of the  $b^*$  versus time curve between the time of zero and infinity, the total area is given by:

$$\text{Area} = \frac{b_o^* B}{V_1}$$

The turnover rate can now be obtained from:

$$V_1 = \frac{b_o^* B}{\text{Area}}$$

Dividing the numerator and denominator by the pool size B:

$$V_1 = \frac{b_o^*}{\int_0^\infty b \, dt}$$

where  $b$  is the specific activity of glutamine in the pool at any time.

It is apparent that if only the area under the monoexponential part of the specific activity decay curve is utilized rather than the entire area, the denominator in the equation above would be too small making the calculated  $V_1$  value too large. Thus, all processes which act to remove or disperse label from the point where it enters the system should be considered if one is to obtain the same values as that obtained by the constant infusion method. In the latter, such processes which occur at multiexponential rates are inherently measured since these processes act continuously on all new label infused into the system.



The reason for the large discrepancy in the values obtained for glutamine turnover compared to the relatively minor discrepancies found in values for glucose turnover may also be apparent. Processes which account for the discrepancy in the values obtained for the turnover of any metabolite include mixing of the label throughout the total pool. The total pool will consist of the plasma pool as well as any tissue pool to which label may be accessible. However, any metabolite which is concentrated within only one pool (i.e., the plasma) will have its label more quickly and simply dispersed than would one which is spread throughout various tissues. Such rapid dispersion of label will make the  $\ln$  specific activity versus time curve approach the monoexponential form more quickly and allow a simple interpretation to be applied. Thus, while the bulk of the free glucose in the body is in the plasma and interstitial fluids (73), the plasma glutamine pool forms less than 1% of the total body glutamine pool (Table 8). Therefore, while the application of the Zilversmit formula gives only minor differences from the constant infusion value for glucose, much larger discrepancies can be expected for glutamine.

The pool size calculated by extrapolation of the linear portion of the  $\ln$  specific activity versus time curve back to zero (Table 6) can be compared to the total pool size of free glutamine in the rat (Table 7). Although the calculated pool size is only half as large as the total body pool, use of the nonlinear part of the curve in the determination of the pool size would give a larger calculated value. It is not known, however, if the calculated value would then be as large as

the total body pool.

(11) Estimation of Errors.

The estimation of the magnitude of the errors involved in the determination of turnover rates has been discussed by Hetenyi and Norwich (37). In an infusion experiment, the S.E.M. of the plateau specific activity divided by the plateau specific activity gives the fractional error in the turnover rate since random errors in the rate of infusion are negligibly small. A regression analysis of the specific activity of the label in the plasma versus time should be done to determine if a steady plateau specific activity has been reached. In single injection experiments, error analysis is much more difficult since the accuracy of the turnover rate obtained depends not only on random errors but on the number of data points and weighting factors attached to each. No simple formula for the determination of fractional errors has been given.

Errors in the value obtained for the turnover rate also arise from recirculation of label, i.e., from the resynthesis of labelled glutamine from labelled breakdown products. When this occurs, the value obtained for the turnover rate is lowered since when the label recirculates, it takes longer for all of the labelled substance to disappear from the system. As already discussed in the Introduction, it was hoped to minimize the recirculation of labelled glutamine in our experiments by the use of 1-<sup>14</sup>C-labelled glutamine. However, it was noted in initial infusion experiments, where <sup>14</sup>C-glutamine radioactivity was determined by a modification of the method of Adam and Simpson (1), that as the infusion went on label in a neutral substance in the plasma samples in-



creased. It was first thought that this substance could be urea, although treatment with urease (70) failed to release  $^{14}\text{CO}_2$  from the incubation mixture after acidification. Similar results were obtained using HCl and  $\text{NaNO}_2$  (64), indicating that the labelled substance was not urea. Preliminary attempts using paper chromatography (24, 78) to determine the identity of this substance were inconclusive. Whether the substance could give rise to  $1\text{-}^{14}\text{C}$ -glutamine is uncertain.

The values for turnover rates of glutamine and other amino acids obtained by other authors have been mentioned in the Introduction. The values obtained in the constant infusion experiments agree well with those reported by Duda and Handler (25). Little difference in turnover rates of  $\text{U-}^{14}\text{C}$ -glutamine has been reported in different acid-base states in sheep (35).

#### B. Measurement of Arterio-Venous Differences Across All Organs

Arterio-venous difference measurements are a direct way of determining how an organ affects metabolites in the blood. Unfortunately, however, the variability found in blood metabolite levels in different animals introduces considerable random errors in the data. Calculated differences in the metabolite levels between different blood vessels must also have a considerable error, and a large number of experiments must be performed to determine significant arterio-venous differences.

In our experiments metabolites were always measured in paired blood vessels from a single rat so that the arterio-venous differences thus calculated contain considerably less random errors. Nevertheless an



arterio-venous difference is usually the small difference between two large numbers and hence the error entailed may be large. For this reason it is quite possible that arterio-venous differences measured across many organs may not reveal statistically significant differences whereas the tissue in question may be actively metabolizing or producing the substance being measured.

Our experiments failed to identify the extra-renal source of the glutamine used by the kidney. Indeed there may be no extra glutamine as the turnover studies indicate, by both techniques, that the whole body turnover of glutamine is unchanged in acidosis. This implies that glutamine that would normally be used extra-renally is diverted to the kidneys in acidosis. An examination of the magnitude of the fluxes involved is instructive. Using the most reliable turnover technique for glutamine, the constant infusion technique, the whole body metabolism of glutamine was estimated to be about  $130 \mu\text{moles/hr/100 g body wt}$  in both normal and acidotic rats (Table 6). Ammonia excretion in such acidotic rats averages about  $2 \text{ mmoles/day/100 g body wt}$  (58). Since only about half of the ammonia formed in the kidney enters the urine (the rest is taken away in the renal venous outflow) and since each glutamine molecule gives rise to two molecules of ammonia it can be calculated that the kidneys of such rats utilize about  $2 \text{ mmoles of glutamine/day/100 g body wt}$  or about  $83 \mu\text{moles/hr/100 g body wt}$ . Thus the renal utilization in acidosis is about two-thirds of the total turnover measured in normal animals. It is certain that an increased turnover of this magnitude would have been detected as can be seen from the relatively small

standard deviations of the estimates of turnover (Table 6). Since no such increase was observed it must be concluded that it did not take place. Hence, we conclude that the extra glutamine utilized by the acidotic kidney is at the expense of glutamine normally utilized by other tissues, i.e., no extra glutamine is formed.

However, our experiments also failed to reveal which tissues used less glutamine. The tissues examined by arterio-venous difference were specifically chosen because of their importance in determining the overall metabolism of the body. Thus, renal blood flow amounts to about 27% of the cardiac output, hepatic + G.I. tract to about 29%, brain to about 15% and muscle (resting) to about 17%. These figures refer to man but the situation in all mammals is probably very similar. Thus, the vessels sampled accounted for 88% of the cardiac output in toto and thus should give a good quantitative picture of glutamine metabolism in vivo. Nevertheless, although the renal uptake was easily evident in the acidotic rats (Table 9), there was no tissue identified which utilized less glutamine. In fact, it can be suggested that no single such tissue exists since it would easily be identified in this analysis. This is so because the blood flows to all of the other regions sampled are either similar in magnitude (liver + G.I. tract) or less than (brain, muscle) the renal blood flow. Thus, any alteration in the metabolism of one of these tissues sufficiently large to account for the renal uptake during acidosis (either a decreased uptake or an increased output) would be sufficiently large to be identified by the arterio-venous difference measurements. Since none was detected it must be assumed that altered

metabolism in no single tissue is responsible for the provision of glutamine to the kidney. The most likely hypothesis is that there is less glutamine utilization by all tissues except the kidney and that the sum of these deficits in utilization is sufficient to supply the extra glutamine required by the acidotic kidney. Since the sparing of glutamine is spread over a number of tissues the altered arterio-venous difference in any one tissue is insufficient to be observed experimentally. The lower extra-renal utilization of glutamine is probably due to the lower blood concentration, which in turn is due to the increased renal extraction.



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